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UTILITY PATENT APPLICATION TRANSMITTAL

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Attorney Docket No. 99,569-A

First Named Inventor Nakayama et al

Express Mail No. EL625499190US

Total Pages

APPLICATION ELEMENTS

1. ☒ Transmittal Form with Fee
2. ☒ Specification (including claims and abstract) [Total Pages 125]
3. ☒ Drawings [Total Sheets 31]
4. ☐ Oath or Declaration [Total Pages]
 - a. ☐ Newly executed
 - b. ☐ Copy from prior application

[Note Boxes 5 and 17 below]

 - i. ☐ Deletion of Inventor(s) Signed statement attached deleting inventor(s) named in the prior application
5. ☐ Incorporation by Reference: The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ Microfiche Computer Program
7. ☒ Nucleotide and/or Amino Acid Sequence Submission
 - a. ☒ Computer Readable Copy
 - b. ☒ Paper Copy
 - c. ☒ Statement verifying above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers
9. ☐ Power of Attorney
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS)
 - ☐ PTO-1449 Form
 - ☐ Copies of IDS Citations
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16. ☒ Other: Title Page

17. ☒ This is a CONTINUING APPLICATION. Please note the following:

- a. ☒ This is a ☒ Continuation ☐ Divisional ☐ Continuation-in-part of prior application Serial No. 60/169,494.
- b. ☐ Cancel in this application original claims ____ of the prior application before calculating the filing fee.
- c. ☐ Amend the specification by inserting before the first line the sentence:
This is a ☐ continuation ☐ divisional ☐ continuation-in-part of application Serial No.
- d. ☒ The prior application is assigned of record to Amgen, Inc.

UTILITY PATENT APPLICATION TRANSMITTAL

Attorney Docket No. 99,569-A

APPLICATION FEES

BASIC FEE

\$ 710.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total Claims	94-20=	74	x \$18.00	\$ 592.00
Independent Claims	8- 3=	5	x \$80.00	\$ 400.00
<input checked="" type="checkbox"/> Multiple Dependent Claims(s) if applicable			+\$270.00	\$ 270.00
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24. CORRESPONDENCE ADDRESS

25. SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Name
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Signature

Date

November 28, 2000

[illegible]

Citizen of Japan

Citizen of the United States of America

Citizen of the United States of America

Citizen of the People's Republic of China

Citizen of the People's Republic of China

Title: Chordin-Like Molecules and Uses Thereof

CHORDIN-LIKE MOLECULES AND USES THEREOF

Field of the Invention

The present invention relates to Chordin-Like (CHL) polypeptides and
5 nucleic acid molecules encoding the same. The invention also relates to selective
binding agents, vectors, host cells, and methods for producing CHL polypeptides.
The invention further relates to pharmaceutical compositions and methods for the
diagnosis, treatment, amelioration, and/or prevention of diseases, disorders, and
conditions associated with CHL polypeptides.

Background of the Invention

Technical advances in the identification, cloning, expression, and
manipulation of nucleic acid molecules and the deciphering of the human genome
have greatly accelerated the discovery of novel therapeutics. Rapid nucleic acid
15 sequencing techniques can now generate sequence information at unprecedented
rates and, coupled with computational analyses, allow the assembly of
overlapping sequences into partial and entire genomes and the identification of
polypeptide-encoding regions. A comparison of a predicted amino acid sequence
against a database compilation of known amino acid sequences allows one to
20 determine the extent of homology to previously identified sequences and/or
structural landmarks. The cloning and expression of a polypeptide-encoding
region of a nucleic acid molecule provides a polypeptide product for structural
and functional analyses. The manipulation of nucleic acid molecules and encoded
polypeptides may confer advantageous properties on a product for use as a
25 therapeutic.

In spite of the significant technical advances in genome research over the
past decade, the potential for the development of novel therapeutics based on the
human genome is still largely unrealized. Many genes encoding potentially
beneficial polypeptide therapeutics or those encoding polypeptides, which may
30 act as "targets" for therapeutic molecules, have still not been identified.

Accordingly, it is an object of the invention to identify novel polypeptides,

and nucleic acid molecules encoding the same, which have diagnostic or therapeutic benefit.

Bone morphogenetic protein (BMP) is a member of the transforming growth factor- β family, which was originally identified as a factor promoting bone formation from a cartridge implant (Wozney *et al.*, 1988, *Science* 242:1528-34; Celeste *et al.*, 1990, *Proc. Nat. Acad. Sci. USA* 87:9843-47). BMP is also known to play an essential role during the early embryogenesis of the frog, the fly, and in mammals. The precise concentration of active BMP seems to be important for the specification of particular cell types (Dale *et al.*, 1992, *Development* 115:573-85; Dosch *et al.*, 1997, *Development* 124:2325-34). An activity gradient of BMP2/4 is observed in, for example, *Xenopus* embryos in which the lowest expression is detected at the dorsal tip and the highest expression at the ventral tip – establishing the dorsoventral axis determination in the embryo. In another example, the control of BMP concentration at specific sites of tissue development suggests a role for BMP in organogenesis. Control of BMP expression is achieved by either localized expression of the BMP gene products or through the influence of the BMP inhibitor chordin (CHD) (Sasai *et al.*, 1994, *Cell* 79:779-90) – or short gastrulation (SOG) (Francois *et al.*, 1994, *Genes Dev.* 8:2602-16).

CHD/SOG is a large secreted protein produced from the Spemann's organizer, the master-controlling region for the dorsoventral axis specification at the gastrulation stage of *Xenopus* embryogenesis. CHD/SOG functions as a dorsalization factor, as does Noggin (Smith and Harland, 1992, *Cell* 70:829-40), which is also secreted from the organizer. The *Drosophila* SOG has a transmembrane domain at its amino-terminus, suggesting that it may be a type II transmembrane protein (Francois *et al.*, 1994, *Genes Dev.* 8:2602-16). It has been proposed that the carboxyl-terminal side (extracellular domain) of the *Drosophila* SOG is cleaved off. However, *Xenopus* CHD (Sasai *et al.*, 1994, *Cell* 79:779-90), Zebrafish CHD (Schulte-Merker *et al.*, 1997, *Nature* 387:862-63), and murine CHD (Pappano *et al.*, 1998, *Genomics* 52:236-39) do not contain the transmembrane domain. Instead, these proteins have a signal peptide, and are

secreted directly. The CHD/SOG polypeptide contains four repeats of the cysteine-rich domain (CR1-4) that is also found in a variety of extracellular matrix proteins such as collagen and thrombospondin.

CHD/SOG is known to bind to one of the ventralizing factors, BMP4 (Piccolo *et al.*, 1996, *Cell* 86:589-98). BMP4 has been shown to be essential for embryonic development of posterior-ventral mesoderm in mice (Winnier *et al.*, 1995, *Genes Dev.* 9:2105-16). The binding of CHD/SOG to BMP4 inhibits BMP4 activity by preventing BMP4 from binding to its receptor (Piccolo *et al.*, 1996, *Cell* 86:589-98). In this respect, the functional relationship between CHD/SOG and BMP4 resembles that between OPG and OPGL, although CHD/SOG is not structurally related to the BMP receptors. The binding affinity of CHD/SOG to BMP4 is specific and tight ($K_d = 3 \times 10^{-10}$ M (Piccolo *et al.*, 1996, *Cell* 86:589-98), and seems to require proteolysis in order to effectuate the release of bound BMP4. This proteolysis is achieved by a specific metalloprotease – Tolloid (TLD) or BMP1 – that cleaves CHD/SOG to liberate either, or both, the first (CR1) and last (CR4) CR motifs (Piccolo *et al.*, 1997, *Cell* 91: 407-16). Whether or not CHD/SOG has other functions or an independent function through its own receptor remains to be determined.

One of the most important roles of CHD/SOG is to establish a BMP4 morphogen gradient (Jones and Smith, 1998, *Dev. Biol.* 194:12-17). BMP4 itself only migrates a short distance and seems to act essentially on the cell autonomously (Jones *et al.*, 1996, *Curr. Biol.* 6:1468-75). In contrast, the BMP4 inhibitors Noggin and CHD/SOG appear to exert a long-range effect, thereby forming an activity gradient of BMP4.

BMPs also play important roles outside of early embryogenesis, for example in the organogenesis of lung, gut, kidney, skin, heart and teeth, as well as in the later stages of embryogenesis (Hogan, 1996, *Genes Dev.* 10:1580-94). Some BMPs are expressed in a very localized fashion while others are expressed widely in a tissue. The importance of the localized action of BMP for organogenesis has been supported by transgenic mouse experiments using constructs by which BMP concentration is artificially elevated throughout the

target tissue. In the case of lung, BMP4 is expressed in the distal tips of epithelium in the developing lung, and when overexpressed with the surfactant protein C promoter, the development of a small lung in which the structural organization (*i.e.*, branching) has been severely disrupted is observed (Bellusci *et al.*, 1996, *Development* 122:1693-702). Since the putative BMP-activity gradient could also be disrupted by the transgene expression, BMPs expressed widely in the tissue could also play a role in the determination of the structural organization of a tissue.

Noggin is another BMP2/4 inhibitor secreted from Spemann's organizer (Zimmerman *et al.*, 1996, *Cell* 86:599-606). The biological role of Noggin and its mode of action are similar to CHD/SOG in *Xenopus*. Although the most notable function of Noggin is, like CHD/SOG, dorsalization, Noggin null-mutant mice have shown a bone phenotype (hyperplasia of chondrocytes) instead of an early embryonic phenotype (McMahon *et al.*, 1998, *Genes Dev.* 12:1438-52; Brunet *et al.*, 1998, *Science* 280:1455-57). This suggests that CHL or even CHD might have a non-dispensable function in the later stage of embryogenesis.

Summary of the Invention

The present invention relates to novel CHL nucleic acid molecules and encoded polypeptides.

The invention provides for an isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

- (a) the nucleotide sequence as set forth in any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11;
- (b) the nucleotide sequence of the DNA insert in any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964;
- (c) a nucleotide sequence encoding the polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;
- (d) a nucleotide sequence which hybridizes under moderately or highly stringent conditions to the complement of any of (a) - (c); and
- (e) a nucleotide sequence complementary to any of (a) - (c).

The invention also provides for an isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide which is at least
5 about 70 percent identical to the polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, wherein the encoded polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(b) a nucleotide sequence encoding an allelic variant or splice variant
10 of the nucleotide sequence as set forth in any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, the nucleotide sequence of the DNA insert in any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964, or (a);

(c) a region of the nucleotide sequence of any of SEQ ID NO: 1, SEQ
ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, the DNA insert in any of ATCC
15 Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964, (a), or (b) encoding a polypeptide fragment of at least about 25 amino acid residues, wherein the polypeptide fragment has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or is antigenic;

(d) a region of the nucleotide sequence of any of SEQ ID NO: 1, SEQ
20 ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, the DNA insert in any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964, or any of (a) - (c) comprising a fragment of at least about 16 nucleotides;

(e) a nucleotide sequence which hybridizes under moderately or highly stringent conditions to the complement of any of (a) - (d); and

25 (f) a nucleotide sequence complementary to any of (a) - (d).

The invention further provides for an isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide as set forth in any of
30 SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one conservative amino acid substitution, wherein the encoded polypeptide has an

activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(b) a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least
5 one amino acid insertion, wherein the encoded polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(c) a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least
10 one amino acid deletion, wherein the encoded polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(d) a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 which has a C-
15 and/or N- terminal truncation, wherein the encoded polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(e) a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least
20 one modification selected from the group consisting of amino acid substitutions, amino acid insertions, amino acid deletions, C-terminal truncation, and N-terminal truncation, wherein the encoded polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(f) a nucleotide sequence of any of (a) - (e) comprising a fragment of
25 at least about 16 nucleotides;

(g) a nucleotide sequence which hybridizes under moderately or highly stringent conditions to the complement of any of (a) - (f); and

(h) a nucleotide sequence complementary to any of (a) - (e).

The present invention provides for an isolated polypeptide comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12; and

5 (b) the amino acid sequence encoded by the DNA insert in any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964.

The invention also provides for an isolated polypeptide comprising the amino acid sequence selected from the group consisting of:

10 (a) the amino acid sequence as set forth in any of SEQ ID NO: 3, SEQ ID NO: 6, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 13, or SEQ ID NO: 14 optionally further comprising an amino-terminal methionine;

(b) an amino acid sequence for an ortholog of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

15 (c) an amino acid sequence which is at least about 70 percent identical to the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

20 (d) a fragment of the amino acid sequence set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 comprising at least about 25 amino acid residues, wherein the fragment has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or is antigenic; and

25 (e) an amino acid sequence for an allelic variant or splice variant of the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, the amino acid sequence encoded by the DNA insert in any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964, or any of (a) - (c).

The invention further provides for an isolated polypeptide comprising the amino acid sequence selected from the group consisting of:

(a) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one conservative amino acid substitution, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(b) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one amino acid insertion, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(c) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one amino acid deletion, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(d) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 which has a C- and/or N- terminal truncation, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12; and

(e) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one modification selected from the group consisting of amino acid substitutions, amino acid insertions, amino acid deletions, C-terminal truncation, and N-terminal truncation, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

Also provided are fusion polypeptides comprising CHL amino acid sequences.

The present invention also provides for an expression vector comprising the isolated nucleic acid molecules as set forth herein, recombinant host cells comprising the recombinant nucleic acid molecules as set forth herein, and a

method of producing a CHL polypeptide comprising culturing the host cells and optionally isolating the polypeptide so produced.

A transgenic non-human animal comprising a nucleic acid molecule encoding a CHL polypeptide is also encompassed by the invention. The CHL
5 nucleic acid molecules are introduced into the animal in a manner that allows expression and increased levels of a CHL polypeptide, which may include increased circulating levels. Alternatively, the CHL nucleic acid molecules are introduced into the animal in a manner that prevents expression of endogenous CHL polypeptide (*i.e.*, generates a transgenic animal possessing a CHL
10 polypeptide gene knockout). The transgenic non-human animal is preferably a mammal, and more preferably a rodent, such as a rat or a mouse.

Also provided are derivatives of the CHL polypeptides of the present invention.

Additionally provided are selective binding agents such as antibodies and
15 peptides capable of specifically binding the CHL polypeptides of the invention. Such antibodies and peptides may be agonistic or antagonistic.

Pharmaceutical compositions comprising the nucleotides, polypeptides, or selective binding agents of the invention and one or more pharmaceutically acceptable formulation agents are also encompassed by the invention. The
20 pharmaceutical compositions are used to provide therapeutically effective amounts of the nucleotides or polypeptides of the present invention. The invention is also directed to methods of using the polypeptides, nucleic acid molecules, and selective binding agents.

The CHL polypeptides and nucleic acid molecules of the present invention
25 may be used to treat, prevent, ameliorate, and/or detect diseases and disorders, including those recited herein.

The present invention also provides a method of assaying test molecules to identify a test molecule that binds to a CHL polypeptide. The method comprises contacting a CHL polypeptide with a test molecule to determine the extent of
30 binding of the test molecule to the polypeptide. The method further comprises determining whether such test molecules are agonists or antagonists of a CHL

polypeptide. The present invention further provides a method of testing the impact of molecules on the expression of CHL polypeptide or on the activity of CHL polypeptide.

Methods of regulating expression and modulating (*i.e.*, increasing or decreasing) levels of a CHL polypeptide are also encompassed by the invention. One method comprises administering to an animal a nucleic acid molecule encoding a CHL polypeptide. In another method, a nucleic acid molecule comprising elements that regulate or modulate the expression of a CHL polypeptide may be administered. Examples of these methods include gene therapy, cell therapy, and anti-sense therapy as further described herein.

In another aspect of the present invention, the CHL polypeptides may be used for identifying receptors thereof ("CHL polypeptide receptors"). Various forms of "expression cloning" have been extensively used to clone receptors for protein ligands. *See, e.g.*, Simonsen and Lodish, 1994, *Trends Pharmacol. Sci.* 15:437-41 and Tartaglia *et al.*, 1995, *Cell* 83:1263-71. The isolation of a CHL polypeptide receptor is useful for identifying or developing novel agonists and antagonists of the CHL polypeptide signaling pathway. Such agonists and antagonists include soluble CHL polypeptide receptors, anti-CHL polypeptide receptor-selective binding agents (such as antibodies and derivatives thereof), small molecules, and antisense oligonucleotides, any of which can be used for treating one or more disease or disorder, including those disclosed herein.

Brief Description of the Figures

Figures 1A-1C illustrate the nucleotide sequence of the murine CHL gene (SEQ ID NO: 1) and the deduced amino acid sequence of murine CHL polypeptide (SEQ ID NO: 2). The predicted signal peptide is indicated (underlined);

Figures 2A-2D illustrate the nucleotide sequence of the rat CHL gene (SEQ ID NO: 4) and the deduced amino acid sequence of rat CHL polypeptide (SEQ ID NO: 5). The predicted signal peptide is indicated (underlined);

Figures 3A-3C illustrate the nucleotide sequence of the human CHL gene (SEQ ID NO: 7) and the deduced amino acid sequence of human CHL polypeptide (SEQ ID NO: 8). The predicted signal peptide is indicated (underlined and/or double-underlined);

5

Figures 4A-4C illustrate the nucleotide sequence of the human CHLd5 gene (SEQ ID NO: 11) and the deduced amino acid sequence of human CHL polypeptide (SEQ ID NO: 12). The predicted signal peptide is indicated (underlined and/or double-underlined);

10

Figure 5 illustrates the location of pro-collagen repeats (CR1-4) and possible BMP1 cleavage sites (*) in murine CHL polypeptide and CHD/SOG;

Figures 6A-6E illustrate an amino acid sequence alignment of human CHL polypeptide (Hchl; SEQ ID NO: 8), human CHLd5 polypeptide (Hchld5; SEQ ID NO: 12), murine CHL polypeptide (Mchl; SEQ ID NO: 2), rat CHL polypeptide (Rchl; SEQ ID NO: 5), murine Chordin (Mchordin; SEQ ID NO: 15), rat Chordin (Rchordin; SEQ ID NO: 16), and human Chordin (Hchordin; SEQ ID NO: 17);

Figures 7A-7B illustrate the expression of murine CHL mRNA and beta-actin as a control in (7A) adult tissues and (7B) embryos;

25

Figure 8 illustrates the expression of human CHL mRNA and beta-actin as a control in adult and fetal human tissues;

Figures 9A-9B illustrate the differential expression of murine CHL mRNA and beta-actin as a control among the stroma cell lines OP9, D3, F4, and F10;

Figure 10 illustrates the expression of murine CHL mRNA as detected by *in situ* hybridization in normal adult mouse lung, liver, stomach, and intestine (BV = blood vessel);

30

Figure 11 illustrates the expression of murine CHL mRNA as detected by *in situ* hybridization in normal adult mouse kidney (boxed area is shown at higher magnification in the lower panel; C = cortex, M = medulla, P = papilla, and G = glomerulus);

Figure 12 illustrates the expression of murine CHL mRNA as detected by *in situ* hybridization in normal embryonic and adult mouse brain;

Figure 13 illustrates the expression of murine CHL mRNA as detected by *in situ* hybridization in normal embryonic and adult mouse bone;

Figures 14A-14C illustrate Western blot analysis of CHL-FLAG polypeptides;

Figures 15A-15B illustrate the secondary axis-forming activity of murine CHL polypeptide;

Figure 16 illustrates the inhibition of Ter119⁺ erythroid cell generation from ES cells *in vitro* by the murine CHL-FLAG polypeptide.

Figure 17 illustrates the results of a BMP-4-dependent cell proliferation and survival assay in which A5-F stromal cells were incubated with different concentrations of BMP-4 protein.

Figure 18 illustrates the results of a BMP-4-dependent cell proliferation and survival assay in which A5-F stromal cells were incubated with a constant concentration of BMP-4 protein and different concentrations of CHL polypeptide.

Detailed Description of the Invention

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All references cited in this application are expressly incorporated by reference herein.

5 Definitions

The terms “CHL gene” or “CHL nucleic acid molecule” or “CHL polynucleotide” refer to a nucleic acid molecule comprising or consisting of a nucleotide sequence as set forth in any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, a nucleotide sequence encoding the polypeptide as set
10 forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, a nucleotide sequence of the DNA insert in any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964, and nucleic acid molecules as defined herein.

The term “CHL polypeptide allelic variant” refers to one of several possible naturally occurring alternate forms of a gene occupying a given locus on
15 a chromosome of an organism or a population of organisms.

The term “CHL polypeptide splice variant” refers to a nucleic acid molecule, usually RNA, which is generated by alternative processing of intron sequences in an RNA transcript of CHL polypeptide amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

20 The term “isolated nucleic acid molecule” refers to a nucleic acid molecule of the invention that (1) has been separated from at least about 50 percent of proteins, lipids, carbohydrates, or other materials with which it is naturally found when total nucleic acid is isolated from the source cells, (2) is not linked to all or a portion of a polynucleotide to which the “isolated nucleic acid
25 molecule” is linked in nature, (3) is operably linked to a polynucleotide which it is not linked to in nature, or (4) does not occur in nature as part of a larger polynucleotide sequence. Preferably, the isolated nucleic acid molecule of the present invention is substantially free from any other contaminating nucleic acid molecule(s) or other contaminants that are found in its natural environment that
30 would interfere with its use in polypeptide production or its therapeutic, diagnostic, prophylactic or research use.

The term "nucleic acid sequence" or "nucleic acid molecule" refers to a DNA or RNA sequence. The term encompasses molecules formed from any of the known base analogs of DNA and RNA such as, but not limited to 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinyl-cytosine, pseudoisocytosine, 5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-iso-pentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyamino-methyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonyl-methyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

The term "vector" is used to refer to any molecule (*e.g.*, nucleic acid, plasmid, or virus) used to transfer coding information to a host cell.

The term "expression vector" refers to a vector that is suitable for transformation of a host cell and contains nucleic acid sequences that direct and/or control the expression of inserted heterologous nucleic acid sequences. Expression includes, but is not limited to, processes such as transcription, translation, and RNA splicing, if introns are present.

The term "operably linked" is used herein to refer to an arrangement of flanking sequences wherein the flanking sequences so described are configured or assembled so as to perform their usual function. Thus, a flanking sequence operably linked to a coding sequence may be capable of effecting the replication, transcription and/or translation of the coding sequence. For example, a coding sequence is operably linked to a promoter when the promoter is capable of directing transcription of that coding sequence. A flanking sequence need not be

contiguous with the coding sequence, so long as it functions correctly. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered “operably linked” to the coding sequence.

5 The term “host cell” is used to refer to a cell which has been transformed, or is capable of being transformed with a nucleic acid sequence and then of expressing a selected gene of interest. The term includes the progeny of the parent cell, whether or not the progeny is identical in morphology or in genetic make-up to the original parent, so long as the selected gene is present.

10 The term “CHL polypeptide” refers to a polypeptide comprising the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 and related polypeptides. Related polypeptides include CHL polypeptide fragments, CHL polypeptide orthologs, CHL polypeptide variants, and CHL polypeptide derivatives, which possess at least one activity of the
15 polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12. CHL polypeptides may be mature polypeptides, as defined herein, and may or may not have an amino-terminal methionine residue, depending on the method by which they are prepared.

 The term “CHL polypeptide fragment” refers to a polypeptide that
20 comprises a truncation at the amino-terminus (with or without a leader sequence) and/or a truncation at the carboxyl-terminus of the polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12. The term “CHL polypeptide fragment” also refers to amino-terminal and/or carboxyl-terminal truncations of CHL polypeptide orthologs, CHL polypeptide derivatives,
25 or CHL polypeptide variants, or to amino-terminal and/or carboxyl-terminal truncations of the polypeptides encoded by CHL polypeptide allelic variants or CHL polypeptide splice variants. CHL polypeptide fragments may result from alternative RNA splicing or from *in vivo* protease activity. Membrane-bound forms of a CHL polypeptide are also contemplated by the present invention. In
30 preferred embodiments, truncations and/or deletions comprise about 10 amino acids, or about 20 amino acids, or about 50 amino acids, or about 75 amino acids,

or about 100 amino acids, or more than about 100 amino acids. The polypeptide fragments so produced will comprise about 25 contiguous amino acids, or about 50 amino acids, or about 75 amino acids, or about 100 amino acids, or about 150 amino acids, or about 200 amino acids. Such CHL polypeptide fragments may optionally comprise an amino-terminal methionine residue. It will be appreciated that such fragments can be used, for example, to generate antibodies to CHL polypeptides.

The term "CHL polypeptide ortholog" refers to a polypeptide from another species that corresponds to CHL polypeptide amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12. For example, mouse and human CHL polypeptides are considered orthologs of each other.

The term "CHL polypeptide variants" refers to CHL polypeptides comprising amino acid sequences having one or more amino acid sequence substitutions, deletions (such as internal deletions and/or CHL polypeptide fragments), and/or additions (such as internal additions and/or CHL fusion polypeptides) as compared to the CHL polypeptide amino acid sequence set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 (with or without a leader sequence). Variants may be naturally occurring (*e.g.*, CHL polypeptide allelic variants, CHL polypeptide orthologs, and CHL polypeptide splice variants) or artificially constructed. Such CHL polypeptide variants may be prepared from the corresponding nucleic acid molecules having a DNA sequence that varies accordingly from the DNA sequence as set forth in any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11. In preferred embodiments, the variants have from 1 to 3, or from 1 to 5, or from 1 to 10, or from 1 to 15, or from 1 to 20, or from 1 to 25, or from 1 to 50, or from 1 to 75, or from 1 to 100, or more than 100 amino acid substitutions, insertions, additions and/or deletions, wherein the substitutions may be conservative, or non-conservative, or any combination thereof.

The term "CHL polypeptide derivatives" refers to the polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12,

CHL polypeptide fragments, CHL polypeptide orthologs, or CHL polypeptide variants, as defined herein, that have been chemically modified. The term “CHL polypeptide derivatives” also refers to the polypeptides encoded by CHL polypeptide allelic variants or CHL polypeptide splice variants, as defined herein,
5 that have been chemically modified.

The term “mature CHL polypeptide” refers to a CHL polypeptide lacking a leader sequence. A mature CHL polypeptide may also include other modifications such as proteolytic processing of the amino-terminus (with or without a leader sequence) and/or the carboxyl-terminus, cleavage of a smaller
10 polypeptide from a larger precursor, N-linked and/or O-linked glycosylation, and the like. Exemplary mature CHL polypeptides are depicted by the amino acid sequences of SEQ ID NO: 3, SEQ ID NO: 6, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 13, and SEQ ID NO: 14.

The term “CHL fusion polypeptide” refers to a fusion of one or more
15 amino acids (such as a heterologous protein or peptide) at the amino- or carboxyl-terminus of the polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, CHL polypeptide fragments, CHL polypeptide orthologs, CHL polypeptide variants, or CHL derivatives, as defined herein. The term “CHL fusion polypeptide” also refers to a fusion of one or more
20 amino acids at the amino- or carboxyl-terminus of the polypeptide encoded by CHL polypeptide allelic variants or CHL polypeptide splice variants, as defined herein.

The term “biologically active CHL polypeptides” refers to CHL polypeptides having at least one activity characteristic of the polypeptide
25 comprising the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12. In addition, a CHL polypeptide may be active as an immunogen; that is, the CHL polypeptide contains at least one epitope to which antibodies may be raised.

The term “isolated polypeptide” refers to a polypeptide of the present
30 invention that (1) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is naturally

found when isolated from the source cell, (2) is not linked (by covalent or noncovalent interaction) to all or a portion of a polypeptide to which the "isolated polypeptide" is linked in nature, (3) is operably linked (by covalent or noncovalent interaction) to a polypeptide with which it is not linked in nature, or
5 (4) does not occur in nature. Preferably, the isolated polypeptide is substantially free from any other contaminating polypeptides or other contaminants that are found in its natural environment that would interfere with its therapeutic, diagnostic, prophylactic or research use.

The term "identity," as known in the art, refers to a relationship between
10 the sequences of two or more polypeptide molecules or two or more nucleic acid molecules, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between nucleic acid molecules or polypeptides, as the case may be, as determined by the match between strings of two or more nucleotide or two or more amino acid sequences. "Identity"
15 measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (*i.e.*, "algorithms").

The term "similarity" is a related concept, but in contrast to "identity," "similarity" refers to a measure of relatedness which includes both identical
20 matches and conservative substitution matches. If two polypeptide sequences have, for example, 10/20 identical amino acids, and the remainder are all non-conservative substitutions, then the percent identity and similarity would both be 50%. If in the same example, there are five more positions where there are conservative substitutions, then the percent identity remains 50%, but the percent
25 similarity would be 75% (15/20). Therefore, in cases where there are conservative substitutions, the percent similarity between two polypeptides will be higher than the percent identity between those two polypeptides.

The term "naturally occurring" or "native" when used in connection with biological materials such as nucleic acid molecules, polypeptides, host cells, and
30 the like, refers to materials which are found in nature and are not manipulated by man. Similarly, "non-naturally occurring" or "non-native" as used herein refers to

a material that is not found in nature or that has been structurally modified or synthesized by man.

The terms “effective amount” and “therapeutically effective amount” each refer to the amount of a CHL polypeptide or CHL nucleic acid molecule used to support an observable level of one or more biological activities of the CHL polypeptides as set forth herein.

The term “pharmaceutically acceptable carrier” or “physiologically acceptable carrier” as used herein refers to one or more formulation materials suitable for accomplishing or enhancing the delivery of the CHL polypeptide, CHL nucleic acid molecule, or CHL selective binding agent as a pharmaceutical composition.

The term “antigen” refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antibody, and additionally capable of being used in an animal to produce antibodies capable of binding to an epitope of that antigen. An antigen may have one or more epitopes.

The term “selective binding agent” refers to a molecule or molecules having specificity for a CHL polypeptide. As used herein, the terms, “specific” and “specificity” refer to the ability of the selective binding agents to bind to human CHL polypeptides and not to bind to human non-CHL polypeptides. It will be appreciated, however, that the selective binding agents may also bind orthologs of the polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, that is, interspecies versions thereof, such as mouse and rat CHL polypeptides.

The term “transduction” is used to refer to the transfer of genes from one bacterium to another, usually by a phage. “Transduction” also refers to the acquisition and transfer of eukaryotic cellular sequences by retroviruses.

The term “transfection” is used to refer to the uptake of foreign or exogenous DNA by a cell, and a cell has been “transfected” when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art and are disclosed herein. *See, e.g., Graham et al., 1973, Virology 52:456; Sambrook et al., Molecular Cloning, A Laboratory*

Manual (Cold Spring Harbor Laboratories, 1989); Davis *et al.*, *Basic Methods in Molecular Biology* (Elsevier, 1986); and Chu *et al.*, 1981, *Gene* 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

5 The term “transformation” as used herein refers to a change in a cell’s genetic characteristics, and a cell has been transformed when it has been modified to contain a new DNA. For example, a cell is transformed where it is genetically modified from its native state. Following transfection or transduction, the transforming DNA may recombine with that of the cell by physically integrating
10 into a chromosome of the cell, may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is considered to have been stably transformed when the DNA is replicated with the division of the cell.

15 Relatedness of Nucleic Acid Molecules and/or Polypeptides

 It is understood that related nucleic acid molecules include allelic or splice variants of the nucleic acid molecule of any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, and include sequences which are complementary to any of the above nucleotide sequences. Related nucleic acid
20 molecules also include a nucleotide sequence encoding a polypeptide comprising or consisting essentially of a substitution, modification, addition and/or deletion of one or more amino acid residues compared to the polypeptide in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12. Such related CHL polypeptides may comprise, for example, an addition and/or a deletion of one or
25 more N-linked or O-linked glycosylation sites or an addition and/or a deletion of one or more cysteine residues.

 Related nucleic acid molecules also include fragments of CHL nucleic acid molecules which encode a polypeptide of at least about 25 contiguous amino acids, or about 50 amino acids, or about 75 amino acids, or about 100 amino
30 acids, or about 150 amino acids, or about 200 amino acids, or more than 200

amino acid residues of the CHL polypeptide of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

In addition, related CHL nucleic acid molecules also include those molecules which comprise nucleotide sequences which hybridize under
5 moderately or highly stringent conditions as defined herein with the fully complementary sequence of the CHL nucleic acid molecule of any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, or of a molecule encoding a polypeptide, which polypeptide comprises the amino acid sequence as shown in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO:
10 12, or of a nucleic acid fragment as defined herein, or of a nucleic acid fragment encoding a polypeptide as defined herein. Hybridization probes may be prepared using the CHL sequences provided herein to screen cDNA, genomic or synthetic DNA libraries for related sequences. Regions of the DNA and/or amino acid sequence of CHL polypeptide that exhibit significant identity to known sequences
15 are readily determined using sequence alignment algorithms as described herein and those regions may be used to design probes for screening.

The term "highly stringent conditions" refers to those conditions that are designed to permit hybridization of DNA strands whose sequences are highly complementary, and to exclude hybridization of significantly mismatched DNAs.
20 Hybridization stringency is principally determined by temperature, ionic strength, and the concentration of denaturing agents such as formamide. Examples of "highly stringent conditions" for hybridization and washing are 0.015 M sodium chloride, 0.0015 M sodium citrate at 65-68°C or 0.015 M sodium chloride, 0.0015 M sodium citrate, and 50% formamide at 42°C. See Sambrook, Fritsch &
25 Maniatis, *Molecular Cloning: A Laboratory Manual* (2nd ed., Cold Spring Harbor Laboratory, 1989); Anderson *et al.*, *Nucleic Acid Hybridisation: A Practical Approach* Ch. 4 (IRL Press Limited).

More stringent conditions (such as higher temperature, lower ionic strength, higher formamide, or other denaturing agent) may also be used –
30 however, the rate of hybridization will be affected. Other agents may be included in the hybridization and washing buffers for the purpose of reducing non-specific

and/or background hybridization. Examples are 0.1% bovine serum albumin, 0.1% polyvinyl-pyrrolidone, 0.1% sodium pyrophosphate, 0.1% sodium dodecylsulfate, NaDodSO₄, (SDS), ficoll, Denhardt's solution, sonicated salmon sperm DNA (or another non-complementary DNA), and dextran sulfate, although
5 other suitable agents can also be used. The concentration and types of these additives can be changed without substantially affecting the stringency of the hybridization conditions. Hybridization experiments are usually carried out at pH 6.8-7.4; however, at typical ionic strength conditions, the rate of hybridization is nearly independent of pH. See Anderson *et al.*, *Nucleic Acid Hybridisation: A*
10 *Practical Approach* Ch. 4 (IRL Press Limited).

Factors affecting the stability of DNA duplex include base composition, length, and degree of base pair mismatch. Hybridization conditions can be adjusted by one skilled in the art in order to accommodate these variables and allow DNAs of different sequence relatedness to form hybrids. The melting
15 temperature of a perfectly matched DNA duplex can be estimated by the following equation:

$$T_m(^{\circ}\text{C}) = 81.5 + 16.6(\log[\text{Na}^+]) + 0.41(\% \text{G+C}) - 600/\text{N} - 0.72(\% \text{formamide})$$

where N is the length of the duplex formed, [Na⁺] is the molar concentration of the sodium ion in the hybridization or washing solution, %G+C is the percentage
20 of (guanine+cytosine) bases in the hybrid. For imperfectly matched hybrids, the melting temperature is reduced by approximately 1°C for each 1% mismatch.

The term "moderately stringent conditions" refers to conditions under which a DNA duplex with a greater degree of base pair mismatching than could occur under "highly stringent conditions" is able to form. Examples of typical
25 "moderately stringent conditions" are 0.015 M sodium chloride, 0.0015 M sodium citrate at 50-65°C or 0.015 M sodium chloride, 0.0015 M sodium citrate, and 20% formamide at 37-50°C. By way of example, "moderately stringent conditions" of 50°C in 0.015 M sodium ion will allow about a 21% mismatch.

It will be appreciated by those skilled in the art that there is no absolute
30 distinction between "highly stringent conditions" and "moderately stringent conditions." For example, at 0.015 M sodium ion (no formamide), the melting

temperature of perfectly matched long DNA is about 71°C. With a wash at 65°C (at the same ionic strength), this would allow for approximately a 6% mismatch. To capture more distantly related sequences, one skilled in the art can simply lower the temperature or raise the ionic strength.

5 A good estimate of the melting temperature in 1M NaCl* for oligonucleotide probes up to about 20nt is given by:

$$T_m = 2^{\circ}\text{C per A-T base pair} + 4^{\circ}\text{C per G-C base pair}$$

*The sodium ion concentration in 6X salt sodium citrate (SSC) is 1M. *See Suggs et al., Developmental Biology Using Purified Genes* 683 (Brown and Fox, eds.,
10 1981).

High stringency washing conditions for oligonucleotides are usually at a temperature of 0-5°C below the T_m of the oligonucleotide in 6X SSC, 0.1% SDS.

In another embodiment, related nucleic acid molecules comprise or consist of a nucleotide sequence that is at least about 70 percent identical to the
15 nucleotide sequence as shown in any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, or comprise or consist essentially of a nucleotide sequence encoding a polypeptide that is at least about 70 percent identical to the polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12. In preferred embodiments, the nucleotide sequences are about
20 75 percent, or about 80 percent, or about 85 percent, or about 90 percent, or about 95, 96, 97, 98, or 99 percent identical to the nucleotide sequence as shown in any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, or the nucleotide sequences encode a polypeptide that is about 75 percent, or about 80 percent, or about 85 percent, or about 90 percent, or about 95, 96, 97, 98, or 99
25 percent identical to the polypeptide sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12. Related nucleic acid molecules encode polypeptides possessing at least one activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

30 Differences in the nucleic acid sequence may result in conservative and/or non-conservative modifications of the amino acid sequence relative to the amino

acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

Conservative modifications to the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 (and the corresponding
5 modifications to the encoding nucleotides) will produce a polypeptide having functional and chemical characteristics similar to those of CHL polypeptides. In contrast, substantial modifications in the functional and/or chemical characteristics of CHL polypeptides may be accomplished by selecting substitutions in the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5,
10 SEQ ID NO: 8, or SEQ ID NO: 12 that differ significantly in their effect on maintaining (a) the structure of the molecular backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

For example, a “conservative amino acid substitution” may involve a
15 substitution of a native amino acid residue with a nonnative residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position. Furthermore, any native residue in the polypeptide may also be substituted with alanine, as has been previously described for “alanine scanning mutagenesis.”

20 Conservative amino acid substitutions also encompass non-naturally occurring amino acid residues that are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics, and other reversed or inverted forms of amino acid moieties.

Naturally occurring residues may be divided into classes based on
25 common side chain properties:

- 1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
- 2) neutral hydrophilic: Cys, Ser, Thr;
- 3) acidic: Asp, Glu;
- 4) basic: Asn, Gln, His, Lys, Arg;
- 30 5) residues that influence chain orientation: Gly, Pro; and
- 6) aromatic: Trp, Tyr, Phe.

For example, non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class. Such substituted residues may be introduced into regions of the human CHL polypeptide that are homologous with non-human CHL polypeptides, or into the non-homologous regions of the molecule.

In making such changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. The hydropathic indices are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte *et al.*, 1982, *J. Mol. Biol.* 157:105-31). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biologically functionally equivalent protein or peptide thereby created is intended for use in immunological embodiments, as in the present case. The greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.*, with a biological property of the protein.

The following hydrophilicity values have been assigned to these amino acid residues: arginine (+3.0); lysine (+3.0); aspartate ($+3.0 \pm 1$); glutamate ($+3.0 \pm 1$); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-

0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred. One may also identify epitopes from primary amino acid sequences on the basis of hydrophilicity. These regions are also referred to as "epitopic core regions."

Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. For example, amino acid substitutions can be used to identify important residues of the CHL polypeptide, or to increase or decrease the affinity of the CHL polypeptides described herein. Exemplary amino acid substitutions are set forth in Table I.

Table I

Amino Acid Substitutions

Original Residues	Exemplary Substitutions	Preferred Substitutions
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln	Gln
Asp	Glu	Glu
Cys	Ser, Ala	Ser
Gln	Asn	Asn
Glu	Asp	Asp
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleucine	Leu

Leu	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, 1,4 Diamino-butyric Acid, Gln, Asn	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Leu
Pro	Ala	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

A skilled artisan will be able to determine suitable variants of the polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 using well-known techniques. For identifying suitable areas of the molecule that may be changed without destroying biological activity, one skilled in the art may target areas not believed to be important for activity. For example, when similar polypeptides with similar activities from the same species or from other species are known, one skilled in the art may compare the amino acid sequence of a CHL polypeptide to such similar polypeptides. With such a comparison, one can identify residues and portions of the molecules that are conserved among similar polypeptides. It will be appreciated that changes in areas of the CHL molecule that are not conserved relative to such similar polypeptides would be less likely to adversely affect the biological activity and/or structure of a CHL polypeptide. One skilled in the art would also know that, even in relatively conserved regions, one may substitute chemically similar amino acids for the naturally occurring residues while retaining activity (conservative amino acid residue substitutions). Therefore, even areas that may be important for

biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

Additionally, one skilled in the art can review structure-function studies
5 identifying residues in similar polypeptides that are important for activity or structure. In view of such a comparison, one can predict the importance of amino acid residues in a CHL polypeptide that correspond to amino acid residues that are important for activity or structure in similar polypeptides. One skilled in the art may opt for chemically similar amino acid substitutions for such predicted
10 important amino acid residues of CHL polypeptides.

One skilled in the art can also analyze the three-dimensional structure and amino acid sequence in relation to that structure in similar polypeptides. In view of such information, one skilled in the art may predict the alignment of amino acid residues of CHL polypeptide with respect to its three dimensional structure. One
15 skilled in the art may choose not to make radical changes to amino acid residues predicted to be on the surface of the protein, since such residues may be involved in important interactions with other molecules. Moreover, one skilled in the art may generate test variants containing a single amino acid substitution at each amino acid residue. The variants could be screened using activity assays known
20 to those with skill in the art. Such variants could be used to gather information about suitable variants. For example, if one discovered that a change to a particular amino acid residue resulted in destroyed, undesirably reduced, or unsuitable activity, variants with such a change would be avoided. In other words, based on information gathered from such routine experiments, one skilled
25 in the art can readily determine the amino acids where further substitutions should be avoided either alone or in combination with other mutations.

A number of scientific publications have been devoted to the prediction of secondary structure. See Moulton, 1996, *Curr. Opin. Biotechnol.* 7:422-27; Chou *et al.*, 1974, *Biochemistry* 13:222-45; Chou *et al.*, 1974, *Biochemistry* 113:211-22;
30 Chou *et al.*, 1978, *Adv. Enzymol. Relat. Areas Mol. Biol.* 47:45-48; Chou *et al.*, 1978, *Ann. Rev. Biochem.* 47:251-276; and Chou *et al.*, 1979, *Biophys. J.* 26:367-

84. Moreover, computer programs are currently available to assist with predicting secondary structure. One method of predicting secondary structure is based upon homology modeling. For example, two polypeptides or proteins which have a sequence identity of greater than 30%, or similarity greater than 40%, often have similar structural topologies. The recent growth of the protein structural database (PDB) has provided enhanced predictability of secondary structure, including the potential number of folds within the structure of a polypeptide or protein. See Holm *et al.*, 1999, *Nucleic Acids Res.* 27:244-47. It has been suggested that there are a limited number of folds in a given polypeptide or protein and that once a critical number of structures have been resolved, structural prediction will become dramatically more accurate (Brenner *et al.*, 1997, *Curr. Opin. Struct. Biol.* 7:369-76).

Additional methods of predicting secondary structure include "threading" (Jones, 1997, *Curr. Opin. Struct. Biol.* 7:377-87; Sippl *et al.*, 1996, *Structure* 4:15-19), "profile analysis" (Bowie *et al.*, 1991, *Science*, 253:164-70; Gribskov *et al.*, 1990, *Methods Enzymol.* 183:146-59; Gribskov *et al.*, 1987, *Proc. Nat. Acad. Sci. U.S.A.* 84:4355-58), and "evolutionary linkage" (See Holm *et al.*, *supra*, and Brenner *et al.*, *supra*).

Preferred CHL polypeptide variants include glycosylation variants wherein the number and/or type of glycosylation sites have been altered compared to the amino acid sequence set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12. In one embodiment, CHL polypeptide variants comprise a greater or a lesser number of N-linked glycosylation sites than the amino acid sequence set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12. An N-linked glycosylation site is characterized by the sequence: Asn-X-Ser or Asn-X-Thr, wherein the amino acid residue designated as X may be any amino acid residue except proline. The substitution of amino acid residues to create this sequence provides a potential new site for the addition of an N-linked carbohydrate chain. Alternatively, substitutions that eliminate this sequence will remove an existing N-linked carbohydrate chain. Also provided is a rearrangement of N-linked carbohydrate chains wherein one or

more N-linked glycosylation sites (typically those that are naturally occurring) are eliminated and one or more new N-linked sites are created. Additional preferred CHL variants include cysteine variants, wherein one or more cysteine residues are deleted or substituted with another amino acid (*e.g.*, serine) as compared to the amino acid sequence set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12. Cysteine variants are useful when CHL polypeptides must be refolded into a biologically active conformation such as after the isolation of insoluble inclusion bodies. Cysteine variants generally have fewer cysteine residues than the native protein, and typically have an even number to minimize interactions resulting from unpaired cysteines.

In other embodiments, related nucleic acid molecules comprise or consist of a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one amino acid insertion and wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one amino acid deletion and wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

Related nucleic acid molecules also comprise or consist of a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 wherein the polypeptide has a carboxyl- and/or amino-terminal truncation and further wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

Related nucleic acid molecules also comprise or consist of a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one modification selected from the group consisting of amino acid substitutions, amino acid insertions, amino acid deletions, carboxyl-terminal truncations, and amino-terminal truncations and wherein the polypeptide has an activity of the

polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

In addition, the polypeptide comprising the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or other CHL polypeptide, may be fused to a homologous polypeptide to form a homodimer or to a heterologous polypeptide to form a heterodimer. Heterologous peptides and polypeptides include, but are not limited to: an epitope to allow for the detection and/or isolation of a CHL fusion polypeptide; a transmembrane receptor protein or a portion thereof, such as an extracellular domain or a transmembrane and intracellular domain; a ligand or a portion thereof which binds to a transmembrane receptor protein; an enzyme or portion thereof which is catalytically active; a polypeptide or peptide which promotes oligomerization, such as a leucine zipper domain; a polypeptide or peptide which increases stability, such as an immunoglobulin constant region; and a polypeptide which has a therapeutic activity different from the polypeptide comprising the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or other CHL polypeptide.

Fusions can be made either at the amino-terminus or at the carboxyl-terminus of the polypeptide comprising the amino acid sequence set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or other CHL polypeptide. Fusions may be direct with no linker or adapter molecule or may be through a linker or adapter molecule. A linker or adapter molecule may be one or more amino acid residues, typically from about 20 to about 50 amino acid residues. A linker or adapter molecule may also be designed with a cleavage site for a DNA restriction endonuclease or for a protease to allow for the separation of the fused moieties. It will be appreciated that once constructed, the fusion polypeptides can be derivatized according to the methods described herein.

In a further embodiment of the invention, the polypeptide comprising the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or other CHL polypeptide, is fused to one or more domains of an Fc region of human IgG. Antibodies comprise two functionally independent

parts, a variable domain known as “Fab,” that binds an antigen, and a constant domain known as “Fc,” that is involved in effector functions such as complement activation and attack by phagocytic cells. An Fc has a long serum half-life, whereas an Fab is short-lived. Capon *et al.*, 1989, *Nature* 337:525-31. When constructed together with a therapeutic protein, an Fc domain can provide longer half-life or incorporate such functions as Fc receptor binding, protein A binding, complement fixation, and perhaps even placental transfer. *Id.* Table II summarizes the use of certain Fc fusions known in the art.

10

Table II

Fc Fusion with Therapeutic Proteins

Form of Fc	Fusion partner	Therapeutic implications	Reference
IgG1	N-terminus of CD30-L	Hodgkin's disease; anaplastic lymphoma; T-cell leukemia	U.S. Patent No. 5,480,981
Murine Fcγ2a	IL-10	anti-inflammatory; transplant rejection	Zheng <i>et al.</i> , 1995, <i>J. Immunol.</i> 154:5590-600
IgG1	TNF receptor	septic shock	Fisher <i>et al.</i> , 1996, <i>N. Engl. J. Med.</i> 334:1697-1702; Van Zee <i>et al.</i> , 1996, <i>J. Immunol.</i> 156:2221-30
IgG, IgA, IgM, or IgE (excluding the first domain)	TNF receptor	inflammation, autoimmune disorders	U.S. Patent No. 5,808,029
IgG1	CD4 receptor	AIDS	Capon <i>et al.</i> , 1989, <i>Nature</i> 337: 525-31
IgG1, IgG3	N-terminus of IL-2	anti-cancer, antiviral	Harvill <i>et al.</i> , 1995, <i>Immunotech.</i> 1:95-105
IgG1	C-terminus of OPG	osteoarthritis; bone density	WO 97/23614
IgG1	N-terminus of leptin	anti-obesity	PCT/US 97/23183, filed December 11, 1997
Human Ig Cγ1	CTLA-4	autoimmune disorders	Linsley, 1991, <i>J. Exp. Med.</i> , 174:561-69

In one example, a human IgG hinge, CH2, and CH3 region may be fused at either the amino-terminus or carboxyl-terminus of the CHL polypeptides using

methods known to the skilled artisan. In another example, a human IgG hinge, CH2, and CH3 region may be fused at either the amino-terminus or carboxyl-terminus of a CHL polypeptide fragment (*e.g.*, the predicted extracellular portion of CHL polypeptide).

5 The resulting CHL fusion polypeptide may be purified by use of a Protein A affinity column. Peptides and proteins fused to an Fc region have been found to exhibit a substantially greater half-life *in vivo* than the unfused counterpart. Also, a fusion to an Fc region allows for dimerization/multimerization of the fusion polypeptide. The Fc region may be a naturally occurring Fc region, or may be
10 altered to improve certain qualities, such as therapeutic qualities, circulation time, or reduced aggregation.

Identity and similarity of related nucleic acid molecules and polypeptides are readily calculated by known methods. Such methods include, but are not limited to those described in *Computational Molecular Biology* (A.M. Lesk, ed.,
15 Oxford University Press 1988); *Biocomputing: Informatics and Genome Projects* (D.W. Smith, ed., Academic Press 1993); *Computer Analysis of Sequence Data* (Part 1, A.M. Griffin and H.G. Griffin, eds., Humana Press 1994); G. von Heinle, *Sequence Analysis in Molecular Biology* (Academic Press 1987); *Sequence Analysis Primer* (M. Gribskov and J. Devereux, eds., M. Stockton Press 1991);
20 and Carillo *et al.*, 1988, *SIAM J. Applied Math.*, 48:1073.

Preferred methods to determine identity and/or similarity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are described in publicly available computer programs. Preferred computer program methods to determine identity and similarity between
25 two sequences include, but are not limited to, the GCG program package, including GAP (Devereux *et al.*, 1984, *Nucleic Acids Res.* 12:387; Genetics Computer Group, University of Wisconsin, Madison, WI), BLASTP, BLASTN, and FASTA (Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-10). The BLASTX program is publicly available from the National Center for Biotechnology
30 Information (NCBI) and other sources (Altschul *et al.*, *BLAST Manual* (NCB

NLM NIH, Bethesda, MD); Altschul *et al.*, 1990, *supra*). The well-known Smith Waterman algorithm may also be used to determine identity.

Certain alignment schemes for aligning two amino acid sequences may result in the matching of only a short region of the two sequences, and this small aligned region may have very high sequence identity even though there is no significant relationship between the two full-length sequences. Accordingly, in a preferred embodiment, the selected alignment method (GAP program) will result in an alignment that spans at least 50 contiguous amino acids of the claimed polypeptide.

For example, using the computer algorithm GAP (Genetics Computer Group, University of Wisconsin, Madison, WI), two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the “matched span,” as determined by the algorithm). A gap opening penalty (which is calculated as 3X the average diagonal; the “average diagonal” is the average of the diagonal of the comparison matrix being used; the “diagonal” is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 0.1X the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix is also used by the algorithm (*see* Dayhoff *et al.*, 5 *Atlas of Protein Sequence and Structure* (Supp. 3 1978)(PAM250 comparison matrix); Henikoff *et al.*, 1992, *Proc. Natl. Acad. Sci USA* 89:10915-19 (BLOSUM 62 comparison matrix)).

Preferred parameters for polypeptide sequence comparison include the following:

Algorithm: Needleman and Wunsch, 1970, *J. Mol. Biol.* 48:443-53;

Comparison matrix: BLOSUM 62 (Henikoff *et al.*, *supra*);

Gap Penalty: 12

Gap Length Penalty: 4

Threshold of Similarity: 0

The GAP program is useful with the above parameters. The aforementioned parameters are the default parameters for polypeptide comparisons (along with no penalty for end gaps) using the GAP algorithm.

- 5 Preferred parameters for nucleic acid molecule sequence comparison include the following:

Algorithm: Needleman and Wunsch, *supra*;

Comparison matrix: matches = +10, mismatch = 0

- 10 Gap Penalty: 50

Gap Length Penalty: 3

The GAP program is also useful with the above parameters. The aforementioned parameters are the default parameters for nucleic acid molecule comparisons.

- 15 Other exemplary algorithms, gap opening penalties, gap extension penalties, comparison matrices, and thresholds of similarity may be used, including those set forth in the Program Manual, Wisconsin Package, Version 9, September, 1997. The particular choices to be made will be apparent to those of skill in the art and will depend on the specific comparison to be made, such as
- 20 DNA-to-DNA, protein-to-protein, protein-to-DNA; and additionally, whether the comparison is between given pairs of sequences (in which case GAP or BestFit are generally preferred) or between one sequence and a large database of sequences (in which case FASTA or BLASTA are preferred).

25 Nucleic Acid Molecules

The nucleic acid molecules encoding a polypeptide comprising the amino acid sequence of a CHL polypeptide can readily be obtained in a variety of ways including, without limitation, chemical synthesis, cDNA or genomic library screening, expression library screening, and/or PCR amplification of cDNA.

- 30 Recombinant DNA methods used herein are generally those set forth in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor

Laboratory Press, 1989) and/or *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., Green Publishers Inc. and Wiley and Sons 1994). The invention provides for nucleic acid molecules as described herein and methods for obtaining such molecules.

5 Where a gene encoding the amino acid sequence of a CHL polypeptide has been identified from one species, all or a portion of that gene may be used as a probe to identify orthologs or related genes from the same species. The probes or primers may be used to screen cDNA libraries from various tissue sources believed to express the CHL polypeptide. In addition, part or all of a nucleic acid
10 molecule having the sequence as set forth in any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11 may be used to screen a genomic library to identify and isolate a gene encoding the amino acid sequence of a CHL polypeptide. Typically, conditions of moderate or high stringency will be employed for screening to minimize the number of false positives obtained from
15 the screening.

 Nucleic acid molecules encoding the amino acid sequence of CHL polypeptides may also be identified by expression cloning which employs the detection of positive clones based upon a property of the expressed protein. Typically, nucleic acid libraries are screened by the binding an antibody or other
20 binding partner (*e.g.*, receptor or ligand) to cloned proteins that are expressed and displayed on a host cell surface. The antibody or binding partner is modified with a detectable label to identify those cells expressing the desired clone.

 Recombinant expression techniques conducted in accordance with the descriptions set forth below may be followed to produce these polynucleotides and to express the encoded polypeptides. For example, by inserting a nucleic acid
25 sequence that encodes the amino acid sequence of a CHL polypeptide into an appropriate vector, one skilled in the art can readily produce large quantities of the desired nucleotide sequence. The sequences can then be used to generate detection probes or amplification primers. Alternatively, a polynucleotide
30 encoding the amino acid sequence of a CHL polypeptide can be inserted into an

expression vector. By introducing the expression vector into an appropriate host, the encoded CHL polypeptide may be produced in large amounts.

Another method for obtaining a suitable nucleic acid sequence is the polymerase chain reaction (PCR). In this method, cDNA is prepared from poly(A)+RNA or total RNA using the enzyme reverse transcriptase. Two primers, typically complementary to two separate regions of cDNA encoding the amino acid sequence of a CHL polypeptide, are then added to the cDNA along with a polymerase such as *Taq* polymerase, and the polymerase amplifies the cDNA region between the two primers.

Another means of preparing a nucleic acid molecule encoding the amino acid sequence of a CHL polypeptide is chemical synthesis using methods well known to the skilled artisan such as those described by Engels *et al.*, 1989, *Angew. Chem. Intl. Ed.* 28:716-34. These methods include, *inter alia*, the phosphotriester, phosphoramidite, and H-phosphonate methods for nucleic acid synthesis. A preferred method for such chemical synthesis is polymer-supported synthesis using standard phosphoramidite chemistry. Typically, the DNA encoding the amino acid sequence of a CHL polypeptide will be several hundred nucleotides in length. Nucleic acids larger than about 100 nucleotides can be synthesized as several fragments using these methods. The fragments can then be ligated together to form the full-length nucleotide sequence of a CHL gene. Usually, the DNA fragment encoding the amino-terminus of the polypeptide will have an ATG, which encodes a methionine residue. This methionine may or may not be present on the mature form of the CHL polypeptide, depending on whether the polypeptide produced in the host cell is designed to be secreted from that cell. Other methods known to the skilled artisan may be used as well.

In certain embodiments, nucleic acid variants contain codons which have been altered for optimal expression of a CHL polypeptide in a given host cell. Particular codon alterations will depend upon the CHL polypeptide and host cell selected for expression. Such "codon optimization" can be carried out by a variety of methods, for example, by selecting codons which are preferred for use in highly expressed genes in a given host cell. Computer algorithms which

incorporate codon frequency tables such as "Eco_high.Cod" for codon preference of highly expressed bacterial genes may be used and are provided by the University of Wisconsin Package Version 9.0 (Genetics Computer Group, Madison, WI). Other useful codon frequency tables include

5 "Celegans_high.cod," "Celegans_low.cod," "Drosophila_high.cod," "Human_high.cod," "Maize_high.cod," and "Yeast_high.cod."

In some cases, it may be desirable to prepare nucleic acid molecules encoding CHL polypeptide variants. Nucleic acid molecules encoding variants may be produced using site directed mutagenesis, PCR amplification, or other

10 appropriate methods, where the primer(s) have the desired point mutations (*see* Sambrook *et al.*, *supra*, and Ausubel *et al.*, *supra*, for descriptions of mutagenesis techniques). Chemical synthesis using methods described by Engels *et al.*, *supra*, may also be used to prepare such variants. Other methods known to the skilled artisan may be used as well.

15 Vectors and Host Cells

A nucleic acid molecule encoding the amino acid sequence of a CHL polypeptide is inserted into an appropriate expression vector using standard ligation techniques. The vector is typically selected to be functional in the

20 particular host cell employed (*i.e.*, the vector is compatible with the host cell machinery such that amplification of the gene and/or expression of the gene can occur). A nucleic acid molecule encoding the amino acid sequence of a CHL polypeptide may be amplified/expressed in prokaryotic, yeast, insect (baculovirus systems) and/or eukaryotic host cells. Selection of the host cell will depend in

25 part on whether a CHL polypeptide is to be post-translationally modified (*e.g.*, glycosylated and/or phosphorylated). If so, yeast, insect, or mammalian host cells are preferable. For a review of expression vectors, *see Meth. Enz.*, vol. 185 (D.V. Goeddel, ed., Academic Press 1990).

Typically, expression vectors used in any of the host cells will contain

30 sequences for plasmid maintenance and for cloning and expression of exogenous nucleotide sequences. Such sequences, collectively referred to as "flanking

sequences” in certain embodiments will typically include one or more of the following nucleotide sequences: a promoter, one or more enhancer sequences, an origin of replication, a transcriptional termination sequence, a complete intron sequence containing a donor and acceptor splice site, a sequence encoding a leader sequence for polypeptide secretion, a ribosome binding site, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element. Each of these sequences is discussed below.

Optionally, the vector may contain a “tag”-encoding sequence, *i.e.*, an oligonucleotide molecule located at the 5’ or 3’ end of the CHL polypeptide coding sequence; the oligonucleotide sequence encodes polyHis (such as hexaHis), or another “tag” such as FLAG, HA (hemagglutinin influenza virus), or *myc* for which commercially available antibodies exist. This tag is typically fused to the polypeptide upon expression of the polypeptide, and can serve as a means for affinity purification of the CHL polypeptide from the host cell. Affinity purification can be accomplished, for example, by column chromatography using antibodies against the tag as an affinity matrix. Optionally, the tag can subsequently be removed from the purified CHL polypeptide by various means such as using certain peptidases for cleavage.

Flanking sequences may be homologous (*i.e.*, from the same species and/or strain as the host cell), heterologous (*i.e.*, from a species other than the host cell species or strain), hybrid (*i.e.*, a combination of flanking sequences from more than one source), or synthetic, or the flanking sequences may be native sequences which normally function to regulate CHL polypeptide expression. As such, the source of a flanking sequence may be any prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the flanking sequence is functional in, and can be activated by, the host cell machinery.

Flanking sequences useful in the vectors of this invention may be obtained by any of several methods well known in the art. Typically, flanking sequences useful herein – other than the CHL gene flanking sequences – will have been

previously identified by mapping and/or by restriction endonuclease digestion and can thus be isolated from the proper tissue source using the appropriate restriction endonucleases. In some cases, the full nucleotide sequence of a flanking sequence may be known. Here, the flanking sequence may be synthesized using the methods described herein for nucleic acid synthesis or cloning.

Where all or only a portion of the flanking sequence is known, it may be obtained using PCR and/or by screening a genomic library with a suitable oligonucleotide and/or flanking sequence fragment from the same or another species. Where the flanking sequence is not known, a fragment of DNA containing a flanking sequence may be isolated from a larger piece of DNA that may contain, for example, a coding sequence or even another gene or genes. Isolation may be accomplished by restriction endonuclease digestion to produce the proper DNA fragment followed by isolation using agarose gel purification, Qiagen® column chromatography (Chatsworth, CA), or other methods known to the skilled artisan. The selection of suitable enzymes to accomplish this purpose will be readily apparent to one of ordinary skill in the art.

An origin of replication is typically a part of those prokaryotic expression vectors purchased commercially, and the origin aids in the amplification of the vector in a host cell. Amplification of the vector to a certain copy number can, in some cases, be important for the optimal expression of a CHL polypeptide. If the vector of choice does not contain an origin of replication site, one may be chemically synthesized based on a known sequence, and ligated into the vector. For example, the origin of replication from the plasmid pBR322 (New England Biolabs, Beverly, MA) is suitable for most gram-negative bacteria and various origins (*e.g.*, SV40, polyoma, adenovirus, vesicular stomatitis virus (VSV), or papillomaviruses such as HPV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (for example, the SV40 origin is often used only because it contains the early promoter).

A transcription termination sequence is typically located 3' of the end of a polypeptide coding region and serves to terminate transcription. Usually, a

transcription termination sequence in prokaryotic cells is a G-C rich fragment followed by a poly-T sequence. While the sequence is easily cloned from a library or even purchased commercially as part of a vector, it can also be readily synthesized using methods for nucleic acid synthesis such as those described herein.

A selectable marker gene element encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, tetracycline, or kanamycin for prokaryotic host cells; (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media. Preferred selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline resistance gene. A neomycin resistance gene may also be used for selection in prokaryotic and eukaryotic host cells.

Other selection genes may be used to amplify the gene that will be expressed. Amplification is the process wherein genes that are in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of suitable selectable markers for mammalian cells include dihydrofolate reductase (DHFR) and thymidine kinase. The mammalian cell transformants are placed under selection pressure wherein only the transformants are uniquely adapted to survive by virtue of the selection gene present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to the amplification of both the selection gene and the DNA that encodes a CHL polypeptide. As a result, increased quantities of CHL polypeptide are synthesized from the amplified DNA.

A ribosome binding site is usually necessary for translation initiation of mRNA and is characterized by a Shine-Dalgarno sequence (prokaryotes) or a Kozak sequence (eukaryotes). The element is typically located 3' to the promoter and 5' to the coding sequence of a CHL polypeptide to be expressed. The Shine-

Dalgarno sequence is varied but is typically a polypurine (*i.e.*, having a high A-G content). Many Shine-Dalgarno sequences have been identified, each of which can be readily synthesized using methods set forth herein and used in a prokaryotic vector.

5 A leader, or signal, sequence may be used to direct a CHL polypeptide out of the host cell. Typically, a nucleotide sequence encoding the signal sequence is positioned in the coding region of a CHL nucleic acid molecule, or directly at the 5' end of a CHL polypeptide coding region. Many signal sequences have been identified, and any of those that are functional in the selected host cell may be
10 used in conjunction with a CHL nucleic acid molecule. Therefore, a signal sequence may be homologous (naturally occurring) or heterologous to the CHL nucleic acid molecule. Additionally, a signal sequence may be chemically synthesized using methods described herein. In most cases, the secretion of a CHL polypeptide from the host cell via the presence of a signal peptide will result
15 in the removal of the signal peptide from the secreted CHL polypeptide. The signal sequence may be a component of the vector, or it may be a part of a CHL nucleic acid molecule that is inserted into the vector.

 Included within the scope of this invention is the use of either a nucleotide sequence encoding a native CHL polypeptide signal sequence joined to a CHL
20 polypeptide coding region or a nucleotide sequence encoding a heterologous signal sequence joined to a CHL polypeptide coding region. The heterologous signal sequence selected should be one that is recognized and processed, *i.e.*, cleaved by a signal peptidase, by the host cell. For prokaryotic host cells that do not recognize and process the native CHL polypeptide signal sequence, the signal
25 sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, or heat-stable enterotoxin II leaders. For yeast secretion, the native CHL polypeptide signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is
30 satisfactory, although other mammalian signal sequences may be suitable.

In some cases, such as where glycosylation is desired in a eukaryotic host cell expression system, one may manipulate the various presequences to improve glycosylation or yield. For example, one may alter the peptidase cleavage site of a particular signal peptide, or add pro-sequences, which also may affect glycosylation. The final protein product may have, in the -1 position (relative to the first amino acid of the mature protein) one or more additional amino acids incident to expression, which may not have been totally removed. For example, the final protein product may have one or two amino acid residues found in the peptidase cleavage site, attached to the amino-terminus. Alternatively, use of some enzyme cleavage sites may result in a slightly truncated form of the desired CHL polypeptide, if the enzyme cuts at such area within the mature polypeptide.

In many cases, transcription of a nucleic acid molecule is increased by the presence of one or more introns in the vector; this is particularly true where a polypeptide is produced in eukaryotic host cells, especially mammalian host cells. The introns used may be naturally occurring within the CHL gene especially where the gene used is a full-length genomic sequence or a fragment thereof. Where the intron is not naturally occurring within the gene (as for most cDNAs), the intron may be obtained from another source. The position of the intron with respect to flanking sequences and the CHL gene is generally important, as the intron must be transcribed to be effective. Thus, when a CHL cDNA molecule is being transcribed, the preferred position for the intron is 3' to the transcription start site and 5' to the poly-A transcription termination sequence. Preferably, the intron or introns will be located on one side or the other (*i.e.*, 5' or 3') of the cDNA such that it does not interrupt the coding sequence. Any intron from any source, including viral, prokaryotic and eukaryotic (plant or animal) organisms, may be used to practice this invention, provided that it is compatible with the host cell into which it is inserted. Also included herein are synthetic introns. Optionally, more than one intron may be used in the vector.

The expression and cloning vectors of the present invention will typically contain a promoter that is recognized by the host organism and operably linked to the molecule encoding the CHL polypeptide. Promoters are untranscribed

sequences located upstream (*i.e.*, 5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription of the structural gene. Promoters are conventionally grouped into one of two classes: inducible promoters and constitutive promoters. Inducible promoters initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, such as the presence or absence of a nutrient or a change in temperature. Constitutive promoters, on the other hand, initiate continual gene product production; that is, there is little or no control over gene expression. A large number of promoters, recognized by a variety of potential host cells, are well known. A suitable promoter is operably linked to the DNA encoding CHL polypeptide by removing the promoter from the source DNA by restriction enzyme digestion and inserting the desired promoter sequence into the vector. The native CHL promoter sequence may be used to direct amplification and/or expression of a CHL nucleic acid molecule. A heterologous promoter is preferred, however, if it permits greater transcription and higher yields of the expressed protein as compared to the native promoter, and if it is compatible with the host cell system that has been selected for use.

Promoters suitable for use with prokaryotic hosts include the beta-lactamase and lactose promoter systems; alkaline phosphatase; a tryptophan (*trp*) promoter system; and hybrid promoters such as the *tac* promoter. Other known bacterial promoters are also suitable. Their sequences have been published, thereby enabling one skilled in the art to ligate them to the desired DNA sequence, using linkers or adapters as needed to supply any useful restriction sites.

Suitable promoters for use with yeast hosts are also well known in the art. Yeast enhancers are advantageously used with yeast promoters. Suitable promoters for use with mammalian host cells are well known and include, but are not limited to, those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, retroviruses, hepatitis-B virus and most preferably Simian Virus 40 (SV40). Other suitable mammalian promoters include

heterologous mammalian promoters, for example, heat-shock promoters and the actin promoter.

Additional promoters which may be of interest in controlling CHL gene expression include, but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-10); the CMV promoter; the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, *Cell* 22:787-97); the herpes thymidine kinase promoter (Wagner *et al.*, 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:1444-45); the regulatory sequences of the metallothionine gene (Brinster *et al.*, 1982, *Nature* 296:39-42); prokaryotic expression vectors such as the beta-lactamase promoter (Villa-Kamaroff *et al.*, 1978, *Proc. Natl. Acad. Sci. U.S.A.*, 75:3727-31); or the tac promoter (DeBoer *et al.*, 1983, *Proc. Natl. Acad. Sci. U.S.A.*, 80:21-25). Also of interest are the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: the elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, *Cell* 38:639-46; Ornitz *et al.*, 1986, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409 (1986); MacDonald, 1987, *Hepatology* 7:425-515); the insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-22); the immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, *Cell* 38:647-58; Adames *et al.*, 1985, *Nature* 318:533-38; Alexander *et al.*, 1987, *Mol. Cell. Biol.*, 7:1436-44); the mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, *Cell* 45:485-95); the albumin gene control region which is active in liver (Pinkert *et al.*, 1987, *Genes and Devel.* 1:268-76); the alpha-feto-protein gene control region which is active in liver (Krumlauf *et al.*, 1985, *Mol. Cell. Biol.*, 5:1639-48; Hammer *et al.*, 1987, *Science* 235:53-58); the alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, *Genes and Devel.* 1:161-71); the beta-globin gene control region which is active in myeloid cells (Mogam *et al.*, 1985, *Nature* 315:338-40; Kollias *et al.*, 1986, *Cell* 46:89-94); the myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, *Cell* 48:703-12); the

myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, *Nature* 314:283-86); and the gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, *Science* 234:1372-78).

5 An enhancer sequence may be inserted into the vector to increase the transcription of a DNA encoding a CHL polypeptide of the present invention by higher eukaryotes. Enhancers are cis-acting elements of DNA, usually about 10-300 bp in length, that act on the promoter to increase transcription. Enhancers are relatively orientation and position independent. They have been found 5' and 3'
10 to the transcription unit. Several enhancer sequences available from mammalian genes are known (*e.g.*, globin, elastase, albumin, alpha-feto-protein and insulin). Typically, however, an enhancer from a virus will be used. The SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers are exemplary enhancing elements for the activation of
15 eukaryotic promoters. While an enhancer may be spliced into the vector at a position 5' or 3' to a CHL nucleic acid molecule, it is typically located at a site 5' from the promoter.

 Expression vectors of the invention may be constructed from a starting vector such as a commercially available vector. Such vectors may or may not
20 contain all of the desired flanking sequences. Where one or more of the flanking sequences described herein are not already present in the vector, they may be individually obtained and ligated into the vector. Methods used for obtaining each of the flanking sequences are well known to one skilled in the art.

 Preferred vectors for practicing this invention are those which are
25 compatible with bacterial, insect, and mammalian host cells. Such vectors include, *inter alia*, pCRII, pCR3, and pcDNA3.1 (Invitrogen, San Diego, CA), pBSII (Stratagene, La Jolla, CA), pET15 (Novagen, Madison, WI), pGEX (Pharmacia Biotech, Piscataway, NJ), pEGFP-N2 (Clontech, Palo Alto, CA), pETL (BlueBacII, Invitrogen), pDSR-alpha (PCT Pub. No. WO 90/14363) and
30 pFastBacDual (Gibco-BRL, Grand Island, NY).

Additional suitable vectors include, but are not limited to, cosmids, plasmids, or modified viruses, but it will be appreciated that the vector system must be compatible with the selected host cell. Such vectors include, but are not limited to plasmids such as Bluescript[®] plasmid derivatives (a high copy number
5 ColE1-based phagemid, Stratagene Cloning Systems, La Jolla CA), PCR cloning plasmids designed for cloning Taq-amplified PCR products (*e.g.*, TOPO[™] TA Cloning[®] Kit, PCR2.1[®] plasmid derivatives, Invitrogen, Carlsbad, CA), and mammalian, yeast or virus vectors such as a baculovirus expression system (pBacPAK plasmid derivatives, Clontech, Palo Alto, CA).

10 After the vector has been constructed and a nucleic acid molecule encoding a CHL polypeptide has been inserted into the proper site of the vector, the completed vector may be inserted into a suitable host cell for amplification and/or polypeptide expression. The transformation of an expression vector for a CHL polypeptide into a selected host cell may be accomplished by well known
15 methods including methods such as transfection, infection, calcium chloride, electroporation, microinjection, lipofection, DEAE-dextran method, or other known techniques. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan, and are set forth, for example, in Sambrook *et al.*, *supra*.

20 Host cells may be prokaryotic host cells (such as *E. coli*) or eukaryotic host cells (such as a yeast, insect, or vertebrate cell). The host cell, when cultured under appropriate conditions, synthesizes a CHL polypeptide which can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). The
25 selection of an appropriate host cell will depend upon various factors, such as desired expression levels, polypeptide modifications that are desirable or necessary for activity (such as glycosylation or phosphorylation) and ease of folding into a biologically active molecule.

A number of suitable host cells are known in the art and many are
30 available from the American Type Culture Collection (ATCC), Manassas, VA. Examples include, but are not limited to, mammalian cells, such as Chinese

hamster ovary cells (CHO), CHO DHFR(-) cells (Urlaub *et al.*, 1980, *Proc. Natl. Acad. Sci. U.S.A.* 97:4216-20), human embryonic kidney (HEK) 293 or 293T cells, or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening, product production, and purification are known in the art. Other suitable mammalian cell lines, are the monkey COS-1 and COS-7 cell lines, and the CV-1 cell line. Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene. Other suitable mammalian cell lines include but are not limited to, mouse neuroblastoma N2A cells, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines. Each of these cell lines is known by and available to those skilled in the art of protein expression.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, DH5 α , DH10, and MC1061) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, *Streptomyces spp.*, and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for the expression of the polypeptides of the present invention. Preferred yeast cells include, for example, *Saccharomyces cerevisiae* and *Pichia pastoris*.

Additionally, where desired, insect cell systems may be utilized in the methods of the present invention. Such systems are described, for example, in Kitts *et al.*, 1993, *Biotechniques*, 14:810-17; Lucklow, 1993, *Curr. Opin. Biotechnol.* 4:564-72; and Lucklow *et al.*, 1993, *J. Virol.*, 67:4566-79. Preferred insect cells are Sf-9 and Hi5 (Invitrogen).

One may also use transgenic animals to express glycosylated CHL polypeptides. For example, one may use a transgenic milk-producing animal (a

cow or goat, for example) and obtain the present glycosylated polypeptide in the animal milk. One may also use plants to produce CHL polypeptides, however, in general, the glycosylation occurring in plants is different from that produced in mammalian cells, and may result in a glycosylated product which is not suitable for human therapeutic use.

Polypeptide Production

Host cells comprising a CHL polypeptide expression vector may be cultured using standard media well known to the skilled artisan. The media will usually contain all nutrients necessary for the growth and survival of the cells. Suitable media for culturing *E. coli* cells include, for example, Luria Broth (LB) and/or Terrific Broth (TB). Suitable media for culturing eukaryotic cells include Roswell Park Memorial Institute medium 1640 (RPMI 1640), Minimal Essential Medium (MEM) and/or Dulbecco's Modified Eagle Medium (DMEM), all of which may be supplemented with serum and/or growth factors as necessary for the particular cell line being cultured. A suitable medium for insect cultures is Grace's medium supplemented with yeastolate, lactalbumin hydrolysate, and/or fetal calf serum as necessary.

Typically, an antibiotic or other compound useful for selective growth of transfected or transformed cells is added as a supplement to the media. The compound to be used will be dictated by the selectable marker element present on the plasmid with which the host cell was transformed. For example, where the selectable marker element is kanamycin resistance, the compound added to the culture medium will be kanamycin. Other compounds for selective growth include ampicillin, tetracycline, and neomycin.

The amount of a CHL polypeptide produced by a host cell can be evaluated using standard methods known in the art. Such methods include, without limitation, Western blot analysis, SDS-polyacrylamide gel electrophoresis, non-denaturing gel electrophoresis, High Performance Liquid Chromatography (HPLC) separation, immunoprecipitation, and/or activity assays such as DNA binding gel shift assays.

If a CHL polypeptide has been designed to be secreted from the host cells, the majority of polypeptide may be found in the cell culture medium. If however, the CHL polypeptide is not secreted from the host cells, it will be present in the cytoplasm and/or the nucleus (for eukaryotic host cells) or in the cytosol (for
5 gram-negative bacteria host cells).

For a CHL polypeptide situated in the host cell cytoplasm and/or nucleus (for eukaryotic host cells) or in the cytosol (for bacterial host cells), the intracellular material (including inclusion bodies for gram-negative bacteria) can be extracted from the host cell using any standard technique known to the skilled
10 artisan. For example, the host cells can be lysed to release the contents of the periplasm/cytoplasm by French press, homogenization, and/or sonication followed by centrifugation.

If a CHL polypeptide has formed inclusion bodies in the cytosol, the inclusion bodies can often bind to the inner and/or outer cellular membranes and
15 thus will be found primarily in the pellet material after centrifugation. The pellet material can then be treated at pH extremes or with a chaotropic agent such as a detergent, guanidine, guanidine derivatives, urea, or urea derivatives in the presence of a reducing agent such as dithiothreitol at alkaline pH or tris carboxyethyl phosphine at acid pH to release, break apart, and solubilize the
20 inclusion bodies. The solubilized CHL polypeptide can then be analyzed using gel electrophoresis, immunoprecipitation, or the like. If it is desired to isolate the CHL polypeptide, isolation may be accomplished using standard methods such as those described herein and in Marston *et al.*, 1990, *Meth. Enz.*, 182:264-75.

In some cases, a CHL polypeptide may not be biologically active upon
25 isolation. Various methods for "refolding" or converting the polypeptide to its tertiary structure and generating disulfide linkages can be used to restore biological activity. Such methods include exposing the solubilized polypeptide to a pH usually above 7 and in the presence of a particular concentration of a chaotrope. The selection of chaotrope is very similar to the choices used for
30 inclusion body solubilization, but usually the chaotrope is used at a lower concentration and is not necessarily the same as chaotropes used for the

solubilization. In most cases the refolding/oxidation solution will also contain a reducing agent or the reducing agent plus its oxidized form in a specific ratio to generate a particular redox potential allowing for disulfide shuffling to occur in the formation of the protein's cysteine bridges. Some of the commonly used
5 redox couples include cysteine/cystamine, glutathione (GSH)/dithiobis GSH, cupric chloride, dithiothreitol(DTT)/dithiane DTT, and 2-2-mercaptoethanol(bME)/dithio-b(ME). In many instances, a cosolvent may be used or may be needed to increase the efficiency of the refolding, and the more common reagents used for this purpose include glycerol, polyethylene glycol of
10 various molecular weights, arginine and the like.

If inclusion bodies are not formed to a significant degree upon expression of a CHL polypeptide, then the polypeptide will be found primarily in the supernatant after centrifugation of the cell homogenate. The polypeptide may be further isolated from the supernatant using methods such as those described
15 herein.

The purification of a CHL polypeptide from solution can be accomplished using a variety of techniques. If the polypeptide has been synthesized such that it contains a tag such as Hexahistidine (CHL polypeptide/hexaHis) or other small peptide such as FLAG (Eastman Kodak Co., New Haven, CT) or *myc* (Invitrogen,
20 Carlsbad, CA) at either its carboxyl- or amino-terminus, it may be purified in a one-step process by passing the solution through an affinity column where the column matrix has a high affinity for the tag.

For example, polyhistidine binds with great affinity and specificity to nickel. Thus, an affinity column of nickel (such as the Qiagen[®] nickel columns)
25 can be used for purification of CHL polypeptide/polyHis. *See, e.g., Current Protocols in Molecular Biology* § 10.11.8 (Ausubel *et al.*, eds., Green Publishers Inc. and Wiley and Sons 1993).

Additionally, CHL polypeptides may be purified through the use of a monoclonal antibody that is capable of specifically recognizing and binding to a
30 CHL polypeptide.

Other suitable procedures for purification include, without limitation, affinity chromatography, immunoaffinity chromatography, ion exchange chromatography, molecular sieve chromatography, HPLC, electrophoresis (including native gel electrophoresis) followed by gel elution, and preparative isoelectric focusing ("Isoprime" machine/technique, Hoefer Scientific, San Francisco, CA). In some cases, two or more purification techniques may be combined to achieve increased purity.

CHL polypeptides may also be prepared by chemical synthesis methods (such as solid phase peptide synthesis) using techniques known in the art such as those set forth by Merrifield *et al.*, 1963, *J. Am. Chem. Soc.* 85:2149; Houghten *et al.*, 1985, *Proc Natl Acad. Sci. USA* 82:5132; and Stewart and Young, *Solid Phase Peptide Synthesis* (Pierce Chemical Co. 1984). Such polypeptides may be synthesized with or without a methionine on the amino-terminus. Chemically synthesized CHL polypeptides may be oxidized using methods set forth in these references to form disulfide bridges. Chemically synthesized CHL polypeptides are expected to have comparable biological activity to the corresponding CHL polypeptides produced recombinantly or purified from natural sources, and thus may be used interchangeably with a recombinant or natural CHL polypeptide.

Another means of obtaining CHL polypeptide is via purification from biological samples such as source tissues and/or fluids in which the CHL polypeptide is naturally found. Such purification can be conducted using methods for protein purification as described herein. The presence of the CHL polypeptide during purification may be monitored, for example, using an antibody prepared against recombinantly produced CHL polypeptide or peptide fragments thereof.

A number of additional methods for producing nucleic acids and polypeptides are known in the art, and the methods can be used to produce polypeptides having specificity for CHL polypeptide. *See, e.g.*, Roberts *et al.*, 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94:12297-303, which describes the production of fusion proteins between an mRNA and its encoded peptide. *See also*, Roberts, 1999, *Curr. Opin. Chem. Biol.* 3:268-73. Additionally, U.S. Patent No. 5,824,469 describes methods for obtaining oligonucleotides capable of carrying out a

specific biological function. The procedure involves generating a heterogeneous pool of oligonucleotides, each having a 5' randomized sequence, a central preselected sequence, and a 3' randomized sequence. The resulting heterogeneous pool is introduced into a population of cells that do not exhibit the
5 desired biological function. Subpopulations of the cells are then screened for those that exhibit a predetermined biological function. From that subpopulation, oligonucleotides capable of carrying out the desired biological function are isolated.

U.S. Patent Nos. 5,763,192; 5,814,476; 5,723,323; and 5,817,483 describe
10 processes for producing peptides or polypeptides. This is done by producing stochastic genes or fragments thereof, and then introducing these genes into host cells which produce one or more proteins encoded by the stochastic genes. The host cells are then screened to identify those clones producing peptides or polypeptides having the desired activity.

Another method for producing peptides or polypeptides is described in
15 PCT/US98/20094 (WO99/15650) filed by Athersys, Inc. Known as "Random Activation of Gene Expression for Gene Discovery" (RAGE-GD), the process involves the activation of endogenous gene expression or over-expression of a gene by *in situ* recombination methods. For example, expression of an
20 endogenous gene is activated or increased by integrating a regulatory sequence into the target cell which is capable of activating expression of the gene by non-homologous or illegitimate recombination. The target DNA is first subjected to radiation, and a genetic promoter inserted. The promoter eventually locates a break at the front of a gene, initiating transcription of the gene. This results in
25 expression of the desired peptide or polypeptide.

It will be appreciated that these methods can also be used to create comprehensive CHL polypeptide expression libraries, which can subsequently be used for high throughput phenotypic screening in a variety of assays, such as biochemical assays, cellular assays, and whole organism assays (*e.g.*, plant,
30 mouse, etc.).

Synthesis

It will be appreciated by those skilled in the art that the nucleic acid and polypeptide molecules described herein may be produced by recombinant and other means.

5

Selective Binding Agents

The term “selective binding agent” refers to a molecule that has specificity for one or more CHL polypeptides. Suitable selective binding agents include, but are not limited to, antibodies and derivatives thereof, polypeptides, and small
10 molecules. Suitable selective binding agents may be prepared using methods known in the art. An exemplary CHL polypeptide selective binding agent of the present invention is capable of binding a certain portion of the CHL polypeptide thereby inhibiting the binding of the polypeptide to a CHL polypeptide receptor.

Selective binding agents such as antibodies and antibody fragments that
15 bind CHL polypeptides are within the scope of the present invention. The antibodies may be polyclonal including monospecific polyclonal; monoclonal (MAbs); recombinant; chimeric; humanized, such as CDR-grafted; human; single chain; and/or bispecific; as well as fragments; variants; or derivatives thereof. Antibody fragments include those portions of the antibody that bind to an epitope
20 on the CHL polypeptide. Examples of such fragments include Fab and F(ab') fragments generated by enzymatic cleavage of full-length antibodies. Other binding fragments include those generated by recombinant DNA techniques, such as the expression of recombinant plasmids containing nucleic acid sequences encoding antibody variable regions.

25 Polyclonal antibodies directed toward a CHL polypeptide generally are produced in animals (*e.g.*, rabbits or mice) by means of multiple subcutaneous or intraperitoneal injections of CHL polypeptide and an adjuvant. It may be useful to conjugate a CHL polypeptide to a carrier protein that is immunogenic in the species to be immunized, such as keyhole limpet hemocyanin, serum, albumin,
30 bovine thyroglobulin, or soybean trypsin inhibitor. Also, aggregating agents such

as alum are used to enhance the immune response. After immunization, the animals are bled and the serum is assayed for anti-CHL antibody titer.

Monoclonal antibodies directed toward CHL polypeptides are produced using any method that provides for the production of antibody molecules by
5 continuous cell lines in culture. Examples of suitable methods for preparing monoclonal antibodies include the hybridoma methods of Kohler *et al.*, 1975, *Nature* 256:495-97 and the human B-cell hybridoma method (Kozbor, 1984, *J. Immunol.* 133:3001; Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications* 51-63 (Marcel Dekker, Inc., 1987). Also provided by the
10 invention are hybridoma cell lines that produce monoclonal antibodies reactive with CHL polypeptides.

Monoclonal antibodies of the invention may be modified for use as therapeutics. One embodiment is a "chimeric" antibody in which a portion of the heavy (H) and/or light (L) chain is identical with or homologous to a
15 corresponding sequence in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is/are identical with or homologous to a corresponding sequence in antibodies derived from another species or belonging to another antibody class or subclass. Also included are fragments of such antibodies, so long as they exhibit
20 the desired biological activity. See U.S. Patent No. 4,816,567; Morrison *et al.*, 1985, *Proc. Natl. Acad. Sci.* 81:6851-55.

In another embodiment, a monoclonal antibody of the invention is a "humanized" antibody. Methods for humanizing non-human antibodies are well known in the art. See U.S. Patent Nos. 5,585,089 and 5,693,762. Generally, a
25 humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. Humanization can be performed, for example, using methods described in the art (Jones *et al.*, 1986, *Nature* 321:522-25; Riechmann *et al.*, 1998, *Nature* 332:323-27; Verhoeyen *et al.*, 1988, *Science* 239:1534-36), by substituting at least a portion of a rodent complementarity-determining region
30 (CDR) for the corresponding regions of a human antibody.

Also encompassed by the invention are human antibodies that bind CHL polypeptides. Using transgenic animals (*e.g.*, mice) that are capable of producing a repertoire of human antibodies in the absence of endogenous immunoglobulin production such antibodies are produced by immunization with a CHL polypeptide antigen (*i.e.*, having at least 6 contiguous amino acids), optionally conjugated to a carrier. *See, e.g.*, Jakobovits *et al.*, 1993, *Proc. Natl. Acad. Sci.* 90:2551-55; Jakobovits *et al.*, 1993, *Nature* 362:255-58; Bruggermann *et al.*, 1993, *Year in Immuno.* 7:33. In one method, such transgenic animals are produced by incapacitating the endogenous loci encoding the heavy and light immunoglobulin chains therein, and inserting loci encoding human heavy and light chain proteins into the genome thereof. Partially modified animals, that is those having less than the full complement of modifications, are then cross-bred to obtain an animal having all of the desired immune system modifications. When administered an immunogen, these transgenic animals produce antibodies with human (rather than, *e.g.*, murine) amino acid sequences, including variable regions which are immunospecific for these antigens. *See* PCT App. Nos. PCT/US96/05928 and PCT/US93/06926. Additional methods are described in U.S. Patent No. 5,545,807, PCT App. Nos. PCT/US91/245 and PCT/GB89/01207, and in European Patent Nos. 546073B1 and 546073A1. Human antibodies can also be produced by the expression of recombinant DNA in host cells or by expression in hybridoma cells as described herein.

In an alternative embodiment, human antibodies can also be produced from phage-display libraries (Hoogenboom *et al.*, 1991, *J. Mol. Biol.* 227:381; Marks *et al.*, 1991, *J. Mol. Biol.* 222:581). These processes mimic immune selection through the display of antibody repertoires on the surface of filamentous bacteriophage, and subsequent selection of phage by their binding to an antigen of choice. One such technique is described in PCT App. No. PCT/US98/17364, which describes the isolation of high affinity and functional agonistic antibodies for MPL- and msk- receptors using such an approach.

Chimeric, CDR grafted, and humanized antibodies are typically produced by recombinant methods. Nucleic acids encoding the antibodies are introduced

into host cells and expressed using materials and procedures described herein. In a preferred embodiment, the antibodies are produced in mammalian host cells, such as CHO cells. Monoclonal (*e.g.*, human) antibodies may be produced by the expression of recombinant DNA in host cells or by expression in hybridoma cells as described herein.

The anti-CHL antibodies of the invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays (Sola, *Monoclonal Antibodies: A Manual of Techniques* 147-158 (CRC Press, Inc., 1987)) for the detection and quantitation of CHL polypeptides. The antibodies will bind CHL polypeptides with an affinity that is appropriate for the assay method being employed.

For diagnostic applications, in certain embodiments, anti-CHL antibodies may be labeled with a detectable moiety. The detectable moiety can be any one that is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , ^{125}I , ^{99}Tc , ^{111}In , or ^{67}Ga ; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; or an enzyme, such as alkaline phosphatase, β -galactosidase, or horseradish peroxidase (Bayer, *et al.*, 1990, *Meth. Enz.* 184:138-63).

Competitive binding assays rely on the ability of a labeled standard (*e.g.*, a CHL polypeptide, or an immunologically reactive portion thereof) to compete with the test sample analyte (an CHL polypeptide) for binding with a limited amount of anti-CHL antibody. The amount of a CHL polypeptide in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies typically are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays typically involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected and/or quantitated. In a sandwich assay, the test sample analyte is

typically bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. *See, e.g.*, U.S. Patent No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assays). For example, one type of sandwich assay is an enzyme-linked immunosorbent assay (ELISA), in which case the detectable moiety is an enzyme.

The selective binding agents, including anti-CHL antibodies, are also useful for *in vivo* imaging. An antibody labeled with a detectable moiety may be administered to an animal, preferably into the bloodstream, and the presence and location of the labeled antibody in the host assayed. The antibody may be labeled with any moiety that is detectable in an animal, whether by nuclear magnetic resonance, radiology, or other detection means known in the art.

Selective binding agents of the invention, including antibodies, may be used as therapeutics. These therapeutic agents are generally agonists or antagonists, in that they either enhance or reduce, respectively, at least one of the biological activities of a CHL polypeptide. In one embodiment, antagonist antibodies of the invention are antibodies or binding fragments thereof which are capable of specifically binding to a CHL polypeptide and which are capable of inhibiting or eliminating the functional activity of a CHL polypeptide *in vivo* or *in vitro*. In preferred embodiments, the selective binding agent, *e.g.*, an antagonist antibody, will inhibit the functional activity of a CHL polypeptide by at least about 50%, and preferably by at least about 80%. In another embodiment, the selective binding agent may be an anti-CHL polypeptide antibody that is capable of interacting with a CHL polypeptide binding partner (a ligand or receptor) thereby inhibiting or eliminating CHL polypeptide activity *in vitro* or *in vivo*. Selective binding agents, including agonist and antagonist anti-CHL polypeptide antibodies, are identified by screening assays that are well known in the art.

The invention also relates to a kit comprising CHL selective binding agents (such as antibodies) and other reagents useful for detecting CHL

polypeptide levels in biological samples. Such reagents may include a detectable label, blocking serum, positive and negative control samples, and detection reagents.

5 Microarrays

It will be appreciated that DNA microarray technology can be utilized in accordance with the present invention. DNA microarrays are miniature, high-density arrays of nucleic acids positioned on a solid support, such as glass. Each cell or element within the array contains numerous copies of a single nucleic acid species that acts as a target for hybridization with a complementary nucleic acid sequence (*e.g.*, mRNA). In expression profiling using DNA microarray technology, mRNA is first extracted from a cell or tissue sample and then converted enzymatically to fluorescently labeled cDNA. This material is hybridized to the microarray and unbound cDNA is removed by washing. The expression of discrete genes represented on the array is then visualized by quantitating the amount of labeled cDNA that is specifically bound to each target nucleic acid molecule. In this way, the expression of thousands of genes can be quantitated in a high throughput, parallel manner from a single sample of biological material.

20 This high throughput expression profiling has a broad range of applications with respect to the CHL molecules of the invention, including, but not limited to: the identification and validation of CHL disease-related genes as targets for therapeutics; molecular toxicology of related CHL molecules and inhibitors thereof; stratification of populations and generation of surrogate markers for clinical trials; and enhancing related CHL polypeptide small molecule drug discovery by aiding in the identification of selective compounds in high throughput screens.

Chemical Derivatives

30 Chemically modified derivatives of CHL polypeptides may be prepared by one skilled in the art, given the disclosures described herein. CHL polypeptide

derivatives are modified in a manner that is different – either in the type or location of the molecules naturally attached to the polypeptide. Derivatives may include molecules formed by the deletion of one or more naturally-attached chemical groups. The polypeptide comprising the amino acid sequence of any of
5 SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or other CHL polypeptide, may be modified by the covalent attachment of one or more polymers. For example, the polymer selected is typically water-soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Included within the scope of suitable
10 polymers is a mixture of polymers. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable.

The polymers each may be of any molecular weight and may be branched or unbranched. The polymers each typically have an average molecular weight of between about 2 kDa to about 100 kDa (the term “about” indicating that in
15 preparations of a water-soluble polymer, some molecules will weigh more, some less, than the stated molecular weight). The average molecular weight of each polymer is preferably between about 5 kDa and about 50 kDa, more preferably between about 12 kDa and about 40 kDa and most preferably between about 20 kDa and about 35 kDa.

Suitable water-soluble polymers or mixtures thereof include, but are not limited to, N-linked or O-linked carbohydrates, sugars, phosphates, polyethylene glycol (PEG) (including the forms of PEG that have been used to derivatize proteins, including mono-(C₁-C₁₀), alkoxy-, or aryloxy-polyethylene glycol), monomethoxy-polyethylene glycol, dextran (such as low molecular weight
20 dextran of, for example, about 6 kD), cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), and polyvinyl alcohol. Also encompassed by the present invention are bifunctional crosslinking molecules
25 which may be used to prepare covalently attached CHL polypeptide multimers.
30

In general, chemical derivatization may be performed under any suitable condition used to react a protein with an activated polymer molecule. Methods for preparing chemical derivatives of polypeptides will generally comprise the steps of: (a) reacting the polypeptide with the activated polymer molecule (such as a reactive ester or aldehyde derivative of the polymer molecule) under conditions whereby the polypeptide comprising the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or other CHL polypeptide, becomes attached to one or more polymer molecules, and (b) obtaining the reaction products. The optimal reaction conditions will be determined based on known parameters and the desired result. For example, the larger the ratio of polymer molecules to protein, the greater the percentage of attached polymer molecule. In one embodiment, the CHL polypeptide derivative may have a single polymer molecule moiety at the amino-terminus. *See, e.g.,* U.S. Patent No. 5,234,784.

The pegylation of a polypeptide may be specifically carried out using any of the pegylation reactions known in the art. Such reactions are described, for example, in the following references: Francis *et al.*, 1992, *Focus on Growth Factors* 3:4-10; European Patent Nos. 0154316 and 0401384; and U.S. Patent No. 4,179,337. For example, pegylation may be carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer) as described herein. For the acylation reactions, a selected polymer should have a single reactive ester group. For reductive alkylation, a selected polymer should have a single reactive aldehyde group. A reactive aldehyde is, for example, polyethylene glycol propionaldehyde, which is water stable, or mono C₁-C₁₀ alkoxy or aryloxy derivatives thereof (*see* U.S. Patent No. 5,252,714).

In another embodiment, CHL polypeptides may be chemically coupled to biotin. The biotin/CHL polypeptide molecules are then allowed to bind to avidin, resulting in tetravalent avidin/biotin/CHL polypeptide molecules. CHL polypeptides may also be covalently coupled to dinitrophenol (DNP) or

trinitrophenol (TNP) and the resulting conjugates precipitated with anti-DNP or anti-TNP-IgM to form decameric conjugates with a valency of 10.

Generally, conditions that may be alleviated or modulated by the administration of the present CHL polypeptide derivatives include those described herein for CHL polypeptides. However, the CHL polypeptide derivatives disclosed herein may have additional activities, enhanced or reduced biological activity, or other characteristics, such as increased or decreased half-life, as compared to the non-derivatized molecules.

10 Genetically Engineered Non-Human Animals

Additionally included within the scope of the present invention are non-human animals such as mice, rats, or other rodents; rabbits, goats, sheep, or other farm animals, in which the genes encoding native CHL polypeptide have been disrupted (*i.e.*, “knocked out”) such that the level of expression of CHL polypeptide is significantly decreased or completely abolished. Such animals may be prepared using techniques and methods such as those described in U.S. Patent No. 5,557,032.

The present invention further includes non-human animals such as mice, rats, or other rodents; rabbits, goats, sheep, or other farm animals, in which either the native form of a CHL gene for that animal or a heterologous CHL gene is over-expressed by the animal, thereby creating a “transgenic” animal. Such transgenic animals may be prepared using well known methods such as those described in U.S. Patent No 5,489,743 and PCT Pub. No. WO 94/28122.

The present invention further includes non-human animals in which the promoter for one or more of the CHL polypeptides of the present invention is either activated or inactivated (*e.g.*, by using homologous recombination methods) to alter the level of expression of one or more of the native CHL polypeptides.

These non-human animals may be used for drug candidate screening. In such screening, the impact of a drug candidate on the animal may be measured. For example, drug candidates may decrease or increase the expression of the CHL gene. In certain embodiments, the amount of CHL polypeptide that is produced

may be measured after the exposure of the animal to the drug candidate. Additionally, in certain embodiments, one may detect the actual impact of the drug candidate on the animal. For example, over-expression of a particular gene may result in, or be associated with, a disease or pathological condition. In such cases, one may test a drug candidate's ability to decrease expression of the gene or its ability to prevent or inhibit a pathological condition. In other examples, the production of a particular metabolic product such as a fragment of a polypeptide, may result in, or be associated with, a disease or pathological condition. In such cases, one may test a drug candidate's ability to decrease the production of such a metabolic product or its ability to prevent or inhibit a pathological condition.

Assaying for Other Modulators of CHL Polypeptide Activity

In some situations, it may be desirable to identify molecules that are modulators, *i.e.*, agonists or antagonists, of the activity of CHL polypeptide. Natural or synthetic molecules that modulate CHL polypeptide may be identified using one or more screening assays, such as those described herein. Such molecules may be administered either in an *ex vivo* manner or in an *in vivo* manner by injection, or by oral delivery, implantation device, or the like.

"Test molecule" refers to a molecule that is under evaluation for the ability to modulate (*i.e.*, increase or decrease) the activity of a CHL polypeptide. Most commonly, a test molecule will interact directly with a CHL polypeptide. However, it is also contemplated that a test molecule may also modulate CHL polypeptide activity indirectly, such as by affecting CHL gene expression, or by binding to a CHL polypeptide binding partner (*e.g.*, receptor or ligand). In one embodiment, a test molecule will bind to a CHL polypeptide with an affinity constant of at least about 10^{-6} M, preferably about 10^{-8} M, more preferably about 10^{-9} M, and even more preferably about 10^{-10} M.

Methods for identifying compounds that interact with CHL polypeptides are encompassed by the present invention. In certain embodiments, a CHL polypeptide is incubated with a test molecule under conditions that permit the interaction of the test molecule with a CHL polypeptide, and the extent of the

interaction is measured. The test molecule can be screened in a substantially purified form or in a crude mixture.

In certain embodiments, a CHL polypeptide agonist or antagonist may be a protein, peptide, carbohydrate, lipid, or small molecular weight molecule that interacts with CHL polypeptide to regulate its activity. Molecules which regulate CHL polypeptide expression include nucleic acids which are complementary to nucleic acids encoding a CHL polypeptide, or are complementary to nucleic acid sequences which direct or control the expression of CHL polypeptide, and which act as anti-sense regulators of expression.

Once a test molecule has been identified as interacting with a CHL polypeptide, the molecule may be further evaluated for its ability to increase or decrease CHL polypeptide activity. The measurement of the interaction of a test molecule with CHL polypeptide may be carried out in several formats, including cell-based binding assays, membrane binding assays, solution-phase assays, and immunoassays. In general, a test molecule is incubated with a CHL polypeptide for a specified period of time, and CHL polypeptide activity is determined by one or more assays for measuring biological activity.

The interaction of test molecules with CHL polypeptides may also be assayed directly using polyclonal or monoclonal antibodies in an immunoassay. Alternatively, modified forms of CHL polypeptides containing epitope tags as described herein may be used in solution and immunoassays.

In the event that CHL polypeptides display biological activity through an interaction with a binding partner (*e.g.*, a receptor or a ligand), a variety of *in vitro* assays may be used to measure the binding of a CHL polypeptide to the corresponding binding partner (such as a selective binding agent, receptor, or ligand). These assays may be used to screen test molecules for their ability to increase or decrease the rate and/or the extent of binding of a CHL polypeptide to its binding partner. In one assay, a CHL polypeptide is immobilized in the wells of a microtiter plate. Radiolabeled CHL polypeptide binding partner (for example, iodinated CHL polypeptide binding partner) and a test molecule can then be added either one at a time (in either order) or simultaneously to the wells.

After incubation, the wells can be washed and counted for radioactivity, using a scintillation counter, to determine the extent to which the binding partner bound to the CHL polypeptide. Typically, a molecule will be tested over a range of concentrations, and a series of control wells lacking one or more elements of the test assays can be used for accuracy in the evaluation of the results. An alternative to this method involves reversing the “positions” of the proteins, *i.e.*, immobilizing CHL polypeptide binding partner to the microtiter plate wells, incubating with the test molecule and radiolabeled CHL polypeptide, and determining the extent of CHL polypeptide binding. *See, e.g., Current Protocols in Molecular Biology*, chap. 18 (Ausubel *et al.*, eds., Green Publishers Inc. and Wiley and Sons 1995).

As an alternative to radiolabeling, a CHL polypeptide or its binding partner may be conjugated to biotin, and the presence of biotinylated protein can then be detected using streptavidin linked to an enzyme, such as horse radish peroxidase (HRP) or alkaline phosphatase (AP), which can be detected colorimetrically, or by fluorescent tagging of streptavidin. An antibody directed to a CHL polypeptide or to a CHL polypeptide binding partner, and which is conjugated to biotin, may also be used for purposes of detection following incubation of the complex with enzyme-linked streptavidin linked to AP or HRP.

A CHL polypeptide or a CHL polypeptide binding partner can also be immobilized by attachment to agarose beads, acrylic beads, or other types of such inert solid phase substrates. The substrate-protein complex can be placed in a solution containing the complementary protein and the test compound. After incubation, the beads can be precipitated by centrifugation, and the amount of binding between a CHL polypeptide and its binding partner can be assessed using the methods described herein. Alternatively, the substrate-protein complex can be immobilized in a column with the test molecule and complementary protein passing through the column. The formation of a complex between a CHL polypeptide and its binding partner can then be assessed using any of the techniques described herein (*e.g.*, radiolabelling or antibody binding).

Another *in vitro* assay that is useful for identifying a test molecule which increases or decreases the formation of a complex between a CHL polypeptide binding protein and a CHL polypeptide binding partner is a surface plasmon resonance detector system such as the BIAcore assay system (Pharmacia, Piscataway, NJ). The BIAcore system is utilized as specified by the manufacturer. This assay essentially involves the covalent binding of either CHL polypeptide or a CHL polypeptide binding partner to a dextran-coated sensor chip that is located in a detector. The test compound and the other complementary protein can then be injected, either simultaneously or sequentially, into the chamber containing the sensor chip. The amount of complementary protein that binds can be assessed based on the change in molecular mass that is physically associated with the dextran-coated side of the sensor chip, with the change in molecular mass being measured by the detector system.

In some cases, it may be desirable to evaluate two or more test compounds together for their ability to increase or decrease the formation of a complex between a CHL polypeptide and a CHL polypeptide binding partner. In these cases, the assays set forth herein can be readily modified by adding such additional test compound(s) either simultaneously with, or subsequent to, the first test compound. The remainder of the steps in the assay are as set forth herein.

In vitro assays such as those described herein may be used advantageously to screen large numbers of compounds for an effect on the formation of a complex between a CHL polypeptide and CHL polypeptide binding partner. The assays may be automated to screen compounds generated in phage display, synthetic peptide, and chemical synthesis libraries.

Compounds which increase or decrease the formation of a complex between a CHL polypeptide and a CHL polypeptide binding partner may also be screened in cell culture using cells and cell lines expressing either CHL polypeptide or CHL polypeptide binding partner. Cells and cell lines may be obtained from any mammal, but preferably will be from human or other primate, canine, or rodent sources. The binding of a CHL polypeptide to cells expressing CHL polypeptide binding partner at the surface is evaluated in the presence or

absence of test molecules, and the extent of binding may be determined by, for example, flow cytometry using a biotinylated antibody to a CHL polypeptide binding partner. Cell culture assays can be used advantageously to further evaluate compounds that score positive in protein binding assays described
5 herein.

Cell cultures can also be used to screen the impact of a drug candidate. For example, drug candidates may decrease or increase the expression of the CHL gene. In certain embodiments, the amount of CHL polypeptide or a CHL polypeptide fragment that is produced may be measured after exposure of the cell
10 culture to the drug candidate. In certain embodiments, one may detect the actual impact of the drug candidate on the cell culture. For example, the over-expression of a particular gene may have a particular impact on the cell culture. In such cases, one may test a drug candidate's ability to increase or decrease the expression of the gene or its ability to prevent or inhibit a particular impact on the
15 cell culture. In other examples, the production of a particular metabolic product such as a fragment of a polypeptide, may result in, or be associated with, a disease or pathological condition. In such cases, one may test a drug candidate's ability to decrease the production of such a metabolic product in a cell culture.

20 Internalizing Proteins

The *tat* protein sequence (from HIV) can be used to internalize proteins into a cell. See, e.g., Falwell *et al.*, 1994, *Proc. Natl. Acad. Sci. U.S.A.* 91:664-68. For example, an 11 amino acid sequence (Y-G-R-K-K-R-R-Q-R-R-R; SEQ ID NO: 18) of the HIV *tat* protein (termed the "protein transduction domain," or TAT
25 PDT) has been described as mediating delivery across the cytoplasmic membrane and the nuclear membrane of a cell. See Schwarze *et al.*, 1999, *Science* 285:1569-72; and Nagahara *et al.*, 1998, *Nat. Med.* 4:1449-52. In these procedures, FITC-constructs (FITC-labeled G-G-G-G-Y-G-R-K-K-R-R-Q-R-R-R; SEQ ID NO: 19), which penetrate tissues following intraperitoneal administration, are prepared, and
30 the binding of such constructs to cells is detected by fluorescence-activated cell sorting (FACS) analysis. Cells treated with a *tat*- β -gal fusion protein will

demonstrate β -gal activity. Following injection, expression of such a construct can be detected in a number of tissues, including liver, kidney, lung, heart, and brain tissue. It is believed that such constructs undergo some degree of unfolding in order to enter the cell, and as such, may require a refolding following entry into the cell.

It will thus be appreciated that the *tat* protein sequence may be used to internalize a desired polypeptide into a cell. For example, using the *tat* protein sequence, a CHL antagonist (such as an anti-CHL selective binding agent, small molecule, soluble receptor, or antisense oligonucleotide) can be administered intracellularly to inhibit the activity of a CHL molecule. As used herein, the term "CHL molecule" refers to both CHL nucleic acid molecules and CHL polypeptides as defined herein. Where desired, the CHL protein itself may also be internally administered to a cell using these procedures. *See also*, Straus, 1999, *Science* 285:1466-67.

Cell Source Identification Using CHL Polypeptide

In accordance with certain embodiments of the invention, it may be useful to be able to determine the source of a certain cell type associated with a CHL polypeptide. For example, it may be useful to determine the origin of a disease or pathological condition as an aid in selecting an appropriate therapy. In certain embodiments, nucleic acids encoding a CHL polypeptide can be used as a probe to identify cells described herein by screening the nucleic acids of the cells with such a probe. In other embodiments, one may use anti-CHL polypeptide antibodies to test for the presence of CHL polypeptide in cells, and thus, determine if such cells are of the types described herein.

CHL Polypeptide Compositions and Administration

Therapeutic compositions are within the scope of the present invention. Such CHL polypeptide pharmaceutical compositions may comprise a therapeutically effective amount of a CHL polypeptide or a CHL nucleic acid molecule in admixture with a pharmaceutically or physiologically acceptable

formulation agent selected for suitability with the mode of administration. Pharmaceutical compositions may comprise a therapeutically effective amount of one or more CHL polypeptide selective binding agents in admixture with a pharmaceutically or physiologically acceptable formulation agent selected for suitability with the mode of administration.

Acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed.

The pharmaceutical composition may contain formulation materials for modifying, maintaining, or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption, or penetration of the composition. Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine, or lysine), antimicrobials, antioxidants (such as ascorbic acid, sodium sulfite, or sodium hydrogen-sulfite), buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates, or other organic acids), bulking agents (such as mannitol or glycine), chelating agents (such as ethylenediamine tetraacetic acid (EDTA)), complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin, or hydroxypropyl-beta-cyclodextrin), fillers, monosaccharides, disaccharides, and other carbohydrates (such as glucose, mannose, or dextrans), proteins (such as serum albumin, gelatin, or immunoglobulins), coloring, flavoring and diluting agents, emulsifying agents, hydrophilic polymers (such as polyvinylpyrrolidone), low molecular weight polypeptides, salt-forming counterions (such as sodium), preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid, or hydrogen peroxide), solvents (such as glycerin, propylene glycol, or polyethylene glycol), sugar alcohols (such as mannitol or sorbitol), suspending agents, surfactants or wetting agents (such as pluronics; PEG; sorbitan esters; polysorbates such as polysorbate 20 or polysorbate 80; triton; tromethamine; lecithin; cholesterol or tyloxapal), stability enhancing agents (such as sucrose or sorbitol), tonicity enhancing agents (such as alkali metal halides – preferably sodium or potassium

chloride – or mannitol sorbitol), delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants. *See Remington's Pharmaceutical Sciences* (18th Ed., A.R. Gennaro, ed., Mack Publishing Company 1990).

5 The optimal pharmaceutical composition will be determined by a skilled artisan depending upon, for example, the intended route of administration, delivery format, and desired dosage. *See, e.g., Remington's Pharmaceutical Sciences, supra.* Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the CHL molecule.

10 The primary vehicle or carrier in a pharmaceutical composition may be either aqueous or non-aqueous in nature. For example, a suitable vehicle or carrier for injection may be water, physiological saline solution, or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. Other exemplary
15 pharmaceutical compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which may further include sorbitol or a suitable substitute. In one embodiment of the present invention, CHL polypeptide compositions may be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents (*Remington's*
20 *Pharmaceutical Sciences, supra*) in the form of a lyophilized cake or an aqueous solution. Further, the CHL polypeptide product may be formulated as a lyophilizate using appropriate excipients such as sucrose.

The CHL polypeptide pharmaceutical compositions can be selected for parenteral delivery. Alternatively, the compositions may be selected for
25 inhalation or for delivery through the digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the skill of the art.

The formulation components are present in concentrations that are acceptable to the site of administration. For example, buffers are used to maintain
30 the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

When parenteral administration is contemplated, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable, aqueous solution comprising the desired CHL molecule in a pharmaceutically acceptable vehicle. A particularly suitable vehicle for parenteral injection is sterile distilled water in which a CHL molecule is formulated as a sterile, isotonic solution, properly preserved. Yet another preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polylactic acid or polyglycolic acid), beads, or liposomes, that provides for the controlled or sustained release of the product which may then be delivered via a depot injection. Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. Other suitable means for the introduction of the desired molecule include implantable drug delivery devices.

In one embodiment, a pharmaceutical composition may be formulated for inhalation. For example, CHL polypeptide may be formulated as a dry powder for inhalation. CHL polypeptide or nucleic acid molecule inhalation solutions may also be formulated with a propellant for aerosol delivery. In yet another embodiment, solutions may be nebulized. Pulmonary administration is further described in PCT Pub. No. WO 94/20069, which describes the pulmonary delivery of chemically modified proteins.

It is also contemplated that certain formulations may be administered orally. In one embodiment of the present invention, CHL polypeptides that are administered in this fashion can be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. For example, a capsule may be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional agents can be included to facilitate absorption of the CHL polypeptide. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

Another pharmaceutical composition may involve an effective quantity of CHL polypeptides in a mixture with non-toxic excipients that are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or another appropriate vehicle, solutions can be prepared in unit-dose form. Suitable
5 excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional CHL polypeptide pharmaceutical compositions will be evident
10 to those skilled in the art, including formulations involving CHL polypeptides in sustained- or controlled-delivery formulations. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. *See, e.g.*, PCT/US93/00829, which describes the
15 controlled release of porous polymeric microparticles for the delivery of pharmaceutical compositions.

Additional examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, *e.g.* films, or microcapsules. Sustained release matrices may include polyesters, hydrogels,
20 polylactides (U.S. Patent No. 3,773,919 and European Patent No. 058481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, 1983, *Biopolymers* 22:547-56), poly(2-hydroxyethyl-methacrylate) (Langer *et al.*, 1981, *J. Biomed. Mater. Res.* 15:167-277 and Langer, 1982, *Chem. Tech.* 12:98-105), ethylene vinyl acetate (Langer *et al.*, *supra*) or poly-D(-)-3-hydroxybutyric
25 acid (European Patent No. 133988). Sustained-release compositions may also include liposomes, which can be prepared by any of several methods known in the art. *See, e.g.*, Eppstein *et al.*, 1985, *Proc. Natl. Acad. Sci. USA* 82:3688-92; and European Patent Nos. 036676, 088046, and 143949.

The CHL pharmaceutical composition to be used for *in vivo* administration
30 typically must be sterile. This may be accomplished by filtration through sterile filtration membranes. Where the composition is lyophilized, sterilization using

this method may be conducted either prior to, or following, lyophilization and reconstitution. The composition for parenteral administration may be stored in lyophilized form or in a solution. In addition, parenteral compositions generally are placed into a container having a sterile access port, for example, an
5 intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Once the pharmaceutical composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. Such formulations may be stored either in a
10 ready-to-use form or in a form (*e.g.*, lyophilized) requiring reconstitution prior to administration.

In a specific embodiment, the present invention is directed to kits for producing a single-dose administration unit. The kits may each contain both a first container having a dried protein and a second container having an aqueous
15 formulation. Also included within the scope of this invention are kits containing single and multi-chambered pre-filled syringes (*e.g.*, liquid syringes and lyosyringes).

The effective amount of a CHL pharmaceutical composition to be employed therapeutically will depend, for example, upon the therapeutic context
20 and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment will thus vary depending, in part, upon the molecule delivered, the indication for which the CHL molecule is being used, the route of administration, and the size (body weight, body surface, or organ size) and condition (the age and general health) of the patient. Accordingly, the clinician
25 may titer the dosage and modify the route of administration to obtain the optimal therapeutic effect. A typical dosage may range from about 0.1 $\mu\text{g/kg}$ to up to about 100 mg/kg or more, depending on the factors mentioned above. In other embodiments, the dosage may range from 0.1 $\mu\text{g/kg}$ up to about 100 mg/kg; or 1 $\mu\text{g/kg}$ up to about 100 mg/kg; or 5 $\mu\text{g/kg}$ up to about 100 mg/kg.

30 The frequency of dosing will depend upon the pharmacokinetic parameters of the CHL molecule in the formulation being used. Typically, a clinician will

administer the composition until a dosage is reached that achieves the desired effect. The composition may therefore be administered as a single dose, as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. Appropriate dosages may be ascertained through use of appropriate dose-response data.

The route of administration of the pharmaceutical composition is in accord with known methods, *e.g.*, orally; through injection by intravenous, intraperitoneal, intracerebral (intraparenchymal), intracerebroventricular, intramuscular, intraocular, intraarterial, intraportal, or intralesional routes; by sustained release systems; or by implantation devices. Where desired, the compositions may be administered by bolus injection or continuously by infusion, or by implantation device.

Alternatively or additionally, the composition may be administered locally via implantation of a membrane, sponge, or other appropriate material onto which the desired molecule has been absorbed or encapsulated. Where an implantation device is used, the device may be implanted into any suitable tissue or organ, and delivery of the desired molecule may be via diffusion, timed-release bolus, or continuous administration.

In some cases, it may be desirable to use CHL polypeptide pharmaceutical compositions in an *ex vivo* manner. In such instances, cells, tissues, or organs that have been removed from the patient are exposed to CHL polypeptide pharmaceutical compositions after which the cells, tissues, or organs are subsequently implanted back into the patient.

In other cases, a CHL polypeptide can be delivered by implanting certain cells that have been genetically engineered, using methods such as those described herein, to express and secrete the CHL polypeptide. Such cells may be animal or human cells, and may be autologous, heterologous, or xenogeneic. Optionally, the cells may be immortalized. In order to decrease the chance of an

immunological response, the cells may be encapsulated to avoid infiltration of surrounding tissues. The encapsulation materials are typically biocompatible, semi-permeable polymeric enclosures or membranes that allow the release of the protein product(s) but prevent the destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissues.

As discussed herein, it may be desirable to treat isolated cell populations (such as stem cells, lymphocytes, red blood cells, chondrocytes, neurons, and the like) with one or more CHL polypeptides. This can be accomplished by exposing the isolated cells to the polypeptide directly, where it is in a form that is permeable to the cell membrane.

Additional embodiments of the present invention relate to cells and methods (e.g., homologous recombination and/or other recombinant production methods) for both the *in vitro* production of therapeutic polypeptides and for the production and delivery of therapeutic polypeptides by gene therapy or cell therapy. Homologous and other recombination methods may be used to modify a cell that contains a normally transcriptionally-silent CHL gene, or an under-expressed gene, and thereby produce a cell which expresses therapeutically efficacious amounts of CHL polypeptides.

Homologous recombination is a technique originally developed for targeting genes to induce or correct mutations in transcriptionally active genes. Kucherlapati, 1989, *Prog. in Nucl. Acid Res. & Mol. Biol.* 36:301. The basic technique was developed as a method for introducing specific mutations into specific regions of the mammalian genome (Thomas *et al.*, 1986, *Cell* 44:419-28; Thomas and Capecchi, 1987, *Cell* 51:503-12; Doetschman *et al.*, 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:8583-87) or to correct specific mutations within defective genes (Doetschman *et al.*, 1987, *Nature* 330:576-78). Exemplary homologous recombination techniques are described in U.S. Patent No. 5,272,071; European Patent Nos. 9193051 and 505500; PCT/US90/07642, and PCT Pub No. WO 91/09955).

Through homologous recombination, the DNA sequence to be inserted into the genome can be directed to a specific region of the gene of interest by

attaching it to targeting DNA. The targeting DNA is a nucleotide sequence that is complementary (homologous) to a region of the genomic DNA. Small pieces of targeting DNA that are complementary to a specific region of the genome are put in contact with the parental strand during the DNA replication process. It is a
5 general property of DNA that has been inserted into a cell to hybridize, and therefore, recombine with other pieces of endogenous DNA through shared homologous regions. If this complementary strand is attached to an oligonucleotide that contains a mutation or a different sequence or an additional nucleotide, it too is incorporated into the newly synthesized strand as a result of
10 the recombination. As a result of the proofreading function, it is possible for the new sequence of DNA to serve as the template. Thus, the transferred DNA is incorporated into the genome.

Attached to these pieces of targeting DNA are regions of DNA that may interact with or control the expression of a CHL polypeptide, *e.g.*, flanking
15 sequences. For example, a promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired CHL polypeptide. The control element controls a portion of the DNA present in the host cell genome. Thus, the
20 expression of the desired CHL polypeptide may be achieved not by transfection of DNA that encodes the CHL gene itself, but rather by the use of targeting DNA (containing regions of homology with the endogenous gene of interest) coupled with DNA regulatory segments that provide the endogenous gene sequence with recognizable signals for transcription of a CHL gene.

25 In an exemplary method, the expression of a desired targeted gene in a cell (*i.e.*, a desired endogenous cellular gene) is altered via homologous recombination into the cellular genome at a preselected site, by the introduction of DNA which includes at least a regulatory sequence, an exon, and a splice donor site. These components are introduced into the chromosomal (genomic) DNA in such a
30 manner that this, in effect, results in the production of a new transcription unit (in which the regulatory sequence, the exon, and the splice donor site present in the

DNA construct are operatively linked to the endogenous gene). As a result of the introduction of these components into the chromosomal DNA, the expression of the desired endogenous gene is altered.

Altered gene expression, as described herein, encompasses activating (or
5 causing to be expressed) a gene which is normally silent (unexpressed) in the cell as obtained, as well as increasing the expression of a gene which is not expressed at physiologically significant levels in the cell as obtained. The embodiments further encompass changing the pattern of regulation or induction such that it is different from the pattern of regulation or induction that occurs in the cell as
10 obtained, and reducing (including eliminating) the expression of a gene which is expressed in the cell as obtained.

One method by which homologous recombination can be used to increase, or cause, CHL polypeptide production from a cell's endogenous CHL gene involves first using homologous recombination to place a recombination sequence
15 from a site-specific recombination system (*e.g.*, Cre/loxP, FLP/FRT) (Sauer, 1994, *Curr. Opin. Biotechnol.*, 5:521-27; Sauer, 1993, *Methods Enzymol.*, 225:890-900) upstream of (*i.e.*, 5' to) the cell's endogenous genomic CHL polypeptide coding region. A plasmid containing a recombination site homologous to the site that was placed just upstream of the genomic CHL
20 polypeptide coding region is introduced into the modified cell line along with the appropriate recombinase enzyme. This recombinase causes the plasmid to integrate, via the plasmid's recombination site, into the recombination site located just upstream of the genomic CHL polypeptide coding region in the cell line (Baubonis and Sauer, 1993, *Nucleic Acids Res.* 21:2025-29; O'Gorman *et al.*,
25 1991, *Science* 251:1351-55). Any flanking sequences known to increase transcription (*e.g.*, enhancer/promoter, intron, translational enhancer), if properly positioned in this plasmid, would integrate in such a manner as to create a new or modified transcriptional unit resulting in *de novo* or increased CHL polypeptide production from the cell's endogenous CHL gene.

30 A further method to use the cell line in which the site specific recombination sequence had been placed just upstream of the cell's endogenous

genomic CHL polypeptide coding region is to use homologous recombination to introduce a second recombination site elsewhere in the cell line's genome. The appropriate recombinase enzyme is then introduced into the two-recombination-site cell line, causing a recombination event (deletion, inversion, and translocation) (Sauer, 1994, *Curr. Opin. Biotechnol.*, 5:521-27; Sauer, 1993, *Methods Enzymol.*, 225:890-900) that would create a new or modified transcriptional unit resulting in *de novo* or increased CHL polypeptide production from the cell's endogenous CHL gene.

An additional approach for increasing, or causing, the expression of CHL polypeptide from a cell's endogenous CHL gene involves increasing, or causing, the expression of a gene or genes (*e.g.*, transcription factors) and/or decreasing the expression of a gene or genes (*e.g.*, transcriptional repressors) in a manner which results in *de novo* or increased CHL polypeptide production from the cell's endogenous CHL gene. This method includes the introduction of a non-naturally occurring polypeptide (*e.g.*, a polypeptide comprising a site specific DNA binding domain fused to a transcriptional factor domain) into the cell such that *de novo* or increased CHL polypeptide production from the cell's endogenous CHL gene results.

The present invention further relates to DNA constructs useful in the method of altering expression of a target gene. In certain embodiments, the exemplary DNA constructs comprise: (a) one or more targeting sequences, (b) a regulatory sequence, (c) an exon, and (d) an unpaired splice-donor site. The targeting sequence in the DNA construct directs the integration of elements (a) - (d) into a target gene in a cell such that the elements (b) - (d) are operatively linked to sequences of the endogenous target gene. In another embodiment, the DNA constructs comprise: (a) one or more targeting sequences, (b) a regulatory sequence, (c) an exon, (d) a splice-donor site, (e) an intron, and (f) a splice-acceptor site, wherein the targeting sequence directs the integration of elements (a) - (f) such that the elements of (b) - (f) are operatively linked to the endogenous gene. The targeting sequence is homologous to the preselected site in the cellular chromosomal DNA with which homologous recombination is to occur. In the

construct, the exon is generally 3' of the regulatory sequence and the splice-donor site is 3' of the exon.

If the sequence of a particular gene is known, such as the nucleic acid sequence of CHL polypeptide presented herein, a piece of DNA that is complementary to a selected region of the gene can be synthesized or otherwise
5 obtained, such as by appropriate restriction of the native DNA at specific recognition sites bounding the region of interest. This piece serves as a targeting sequence upon insertion into the cell and will hybridize to its homologous region within the genome. If this hybridization occurs during DNA replication, this piece
10 of DNA, and any additional sequence attached thereto, will act as an Okazaki fragment and will be incorporated into the newly synthesized daughter strand of DNA. The present invention, therefore, includes nucleotides encoding a CHL polypeptide, which nucleotides may be used as targeting sequences.

CHL polypeptide cell therapy, *e.g.*, the implantation of cells producing
15 CHL polypeptides, is also contemplated. This embodiment involves implanting cells capable of synthesizing and secreting a biologically active form of CHL polypeptide. Such CHL polypeptide-producing cells can be cells that are natural producers of CHL polypeptides or may be recombinant cells whose ability to produce CHL polypeptides has been augmented by transformation with a gene
20 encoding the desired CHL polypeptide or with a gene augmenting the expression of CHL polypeptide. Such a modification may be accomplished by means of a vector suitable for delivering the gene as well as promoting its expression and secretion. In order to minimize a potential immunological reaction in patients being administered a CHL polypeptide, as may occur with the administration of a
25 polypeptide of a foreign species, it is preferred that the natural cells producing CHL polypeptide be of human origin and produce human CHL polypeptide. Likewise, it is preferred that the recombinant cells producing CHL polypeptide be transformed with an expression vector containing a gene encoding a human CHL polypeptide.

30 Implanted cells may be encapsulated to avoid the infiltration of surrounding tissue. Human or non-human animal cells may be implanted in

patients in biocompatible, semipermeable polymeric enclosures or membranes that allow the release of CHL polypeptide, but that prevent the destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissue. Alternatively, the patient's own cells, transformed to produce
5 CHL polypeptides *ex vivo*, may be implanted directly into the patient without such encapsulation.

Techniques for the encapsulation of living cells are known in the art, and the preparation of the encapsulated cells and their implantation in patients may be routinely accomplished. For example, Baetge *et al.* (PCT Pub. No. WO 95/05452
10 and PCT/US94/09299) describe membrane capsules containing genetically engineered cells for the effective delivery of biologically active molecules. The capsules are biocompatible and are easily retrievable. The capsules encapsulate cells transfected with recombinant DNA molecules comprising DNA sequences coding for biologically active molecules operatively linked to promoters that are
15 not subject to down-regulation *in vivo* upon implantation into a mammalian host. The devices provide for the delivery of the molecules from living cells to specific sites within a recipient. In addition, *see* U.S. Patent Nos. 4,892,538; 5,011,472; and 5,106,627. A system for encapsulating living cells is described in PCT Pub. No. WO 91/10425 (Aebischer *et al.*). *See also*, PCT Pub. No. WO 91/10470
20 (Aebischer *et al.*); Winn *et al.*, 1991, *Exper. Neurol.* 113:322-29; Aebischer *et al.*, 1991, *Exper. Neurol.* 111:269-75; and Tresco *et al.*, 1992, *ASAIO* 38:17-23.

In vivo and *in vitro* gene therapy delivery of CHL polypeptides is also envisioned. One example of a gene therapy technique is to use the CHL gene (either genomic DNA, cDNA, and/or synthetic DNA) encoding a CHL
25 polypeptide which may be operably linked to a constitutive or inducible promoter to form a "gene therapy DNA construct." The promoter may be homologous or heterologous to the endogenous CHL gene, provided that it is active in the cell or tissue type into which the construct will be inserted. Other components of the gene therapy DNA construct may optionally include DNA molecules designed for
30 site-specific integration (*e.g.*, endogenous sequences useful for homologous recombination), tissue-specific promoters, enhancers or silencers, DNA molecules

capable of providing a selective advantage over the parent cell, DNA molecules useful as labels to identify transformed cells, negative selection systems, cell specific binding agents (as, for example, for cell targeting), cell-specific internalization factors, transcription factors enhancing expression from a vector, and factors enabling vector production.

A gene therapy DNA construct can then be introduced into cells (either *ex vivo* or *in vivo*) using viral or non-viral vectors. One means for introducing the gene therapy DNA construct is by means of viral vectors as described herein. Certain vectors, such as retroviral vectors, will deliver the DNA construct to the chromosomal DNA of the cells, and the gene can integrate into the chromosomal DNA. Other vectors will function as episomes, and the gene therapy DNA construct will remain in the cytoplasm.

In yet other embodiments, regulatory elements can be included for the controlled expression of the CHL gene in the target cell. Such elements are turned on in response to an appropriate effector. In this way, a therapeutic polypeptide can be expressed when desired. One conventional control means involves the use of small molecule dimerizers or rapalogs to dimerize chimeric proteins which contain a small molecule-binding domain and a domain capable of initiating a biological process, such as a DNA-binding protein or transcriptional activation protein (*see* PCT Pub. Nos. WO 96/41865, WO 97/31898, and WO 97/31899). The dimerization of the proteins can be used to initiate transcription of the transgene.

An alternative regulation technology uses a method of storing proteins expressed from the gene of interest inside the cell as an aggregate or cluster. The gene of interest is expressed as a fusion protein that includes a conditional aggregation domain that results in the retention of the aggregated protein in the endoplasmic reticulum. The stored proteins are stable and inactive inside the cell. The proteins can be released, however, by administering a drug (*e.g.*, small molecule ligand) that removes the conditional aggregation domain and thereby specifically breaks apart the aggregates or clusters so that the proteins may be

secreted from the cell. See Aridor *et al.*, 2000, *Science* 287:816-17 and Rivera *et al.*, 2000, *Science* 287:826-30.

Other suitable control means or gene switches include, but are not limited to, the systems described herein. Mifepristone (RU486) is used as a progesterone antagonist. The binding of a modified progesterone receptor ligand-binding domain to the progesterone antagonist activates transcription by forming a dimer of two transcription factors that then pass into the nucleus to bind DNA. The ligand-binding domain is modified to eliminate the ability of the receptor to bind to the natural ligand. The modified steroid hormone receptor system is further described in U.S. Patent No. 5,364,791 and PCT Pub. Nos. WO 96/40911 and WO 97/10337.

Yet another control system uses ecdysone (a fruit fly steroid hormone) which binds to and activates an ecdysone receptor (cytoplasmic receptor). The receptor then translocates to the nucleus to bind a specific DNA response element (promoter from ecdysone-responsive gene). The ecdysone receptor includes a transactivation domain, DNA-binding domain, and ligand-binding domain to initiate transcription. The ecdysone system is further described in U.S. Patent No. 5,514,578 and PCT Pub. Nos. WO 97/38117, WO 96/37609, and WO 93/03162.

Another control means uses a positive tetracycline-controllable transactivator. This system involves a mutated tet repressor protein DNA-binding domain (mutated tet R-4 amino acid changes which resulted in a reverse tetracycline-regulated transactivator protein, *i.e.*, it binds to a tet operator in the presence of tetracycline) linked to a polypeptide which activates transcription. Such systems are described in U.S. Patent Nos. 5,464,758, 5,650,298, and 5,654,168.

Additional expression control systems and nucleic acid constructs are described in U.S. Patent Nos. 5,741,679 and 5,834,186, to Innovir Laboratories Inc.

In vivo gene therapy may be accomplished by introducing the gene encoding CHL polypeptide into cells via local injection of a CHL nucleic acid molecule or by other appropriate viral or non-viral delivery vectors. Hefti 1994,

Neurobiology 25:1418-35. For example, a nucleic acid molecule encoding a CHL polypeptide may be contained in an adeno-associated virus (AAV) vector for delivery to the targeted cells (*see, e.g.*, Johnson, PCT Pub. No. WO 95/34670; PCT App. No. PCT/US95/07178). The recombinant AAV genome typically
5 contains AAV inverted terminal repeats flanking a DNA sequence encoding a CHL polypeptide operably linked to functional promoter and polyadenylation sequences.

Alternative suitable viral vectors include, but are not limited to, retrovirus, adenovirus, herpes simplex virus, lentivirus, hepatitis virus, parvovirus,
10 papovavirus, poxvirus, alphavirus, coronavirus, rhabdovirus, paramyxovirus, and papilloma virus vectors. U.S. Patent No. 5,672,344 describes an *in vivo* viral-mediated gene transfer system involving a recombinant neurotrophic HSV-1 vector. U.S. Patent No. 5,399,346 provides examples of a process for providing a patient with a therapeutic protein by the delivery of human cells which have been
15 treated *in vitro* to insert a DNA segment encoding a therapeutic protein. Additional methods and materials for the practice of gene therapy techniques are described in U.S. Patent Nos. 5,631,236 (involving adenoviral vectors), 5,672,510 (involving retroviral vectors), 5,635,399 (involving retroviral vectors expressing cytokines).

20 Nonviral delivery methods include, but are not limited to, liposome-mediated transfer, naked DNA delivery (direct injection), receptor-mediated transfer (ligand-DNA complex), electroporation, calcium phosphate precipitation, and microparticle bombardment (*e.g.*, gene gun). Gene therapy materials and methods may also include inducible promoters, tissue-specific enhancer-
25 promoters, DNA sequences designed for site-specific integration, DNA sequences capable of providing a selective advantage over the parent cell, labels to identify transformed cells, negative selection systems and expression control systems (safety measures), cell-specific binding agents (for cell targeting), cell-specific internalization factors, and transcription factors to enhance expression by a vector
30 as well as methods of vector manufacture. Such additional methods and materials for the practice of gene therapy techniques are described in U.S. Patent Nos.

4,970,154 (involving electroporation techniques), 5,679,559 (describing a lipoprotein-containing system for gene delivery), 5,676,954 (involving liposome carriers), 5,593,875 (describing methods for calcium phosphate transfection), and 4,945,050 (describing a process wherein biologically active particles are propelled
5 at cells at a speed whereby the particles penetrate the surface of the cells and become incorporated into the interior of the cells), and PCT Pub. No. WO 96/40958 (involving nuclear ligands).

It is also contemplated that CHL gene therapy or cell therapy can further include the delivery of one or more additional polypeptide(s) in the same or a
10 different cell(s). Such cells may be separately introduced into the patient, or the cells may be contained in a single implantable device, such as the encapsulating membrane described above, or the cells may be separately modified by means of viral vectors.

A means to increase endogenous CHL polypeptide expression in a cell via
15 gene therapy is to insert one or more enhancer elements into the CHL polypeptide promoter, where the enhancer elements can serve to increase transcriptional activity of the CHL gene. The enhancer elements used will be selected based on the tissue in which one desires to activate the gene – enhancer elements known to confer promoter activation in that tissue will be selected. For example, if a gene
20 encoding a CHL polypeptide is to be “turned on” in T-cells, the *lck* promoter enhancer element may be used. Here, the functional portion of the transcriptional element to be added may be inserted into a fragment of DNA containing the CHL polypeptide promoter (and optionally, inserted into a vector and/or 5’ and/or 3’ flanking sequences) using standard cloning techniques. This construct, known as
25 a “homologous recombination construct,” can then be introduced into the desired cells either *ex vivo* or *in vivo*.

Gene therapy also can be used to decrease CHL polypeptide expression by modifying the nucleotide sequence of the endogenous promoter. Such modification is typically accomplished via homologous recombination methods.
30 For example, a DNA molecule containing all or a portion of the promoter of the CHL gene selected for inactivation can be engineered to remove and/or replace

pieces of the promoter that regulate transcription. For example, the TATA box and/or the binding site of a transcriptional activator of the promoter may be deleted using standard molecular biology techniques; such deletion can inhibit promoter activity thereby repressing the transcription of the corresponding CHL gene. The deletion of the TATA box or the transcription activator binding site in the promoter may be accomplished by generating a DNA construct comprising all or the relevant portion of the CHL polypeptide promoter (from the same or a related species as the CHL gene to be regulated) in which one or more of the TATA box and/or transcriptional activator binding site nucleotides are mutated via substitution, deletion and/or insertion of one or more nucleotides. As a result, the TATA box and/or activator binding site has decreased activity or is rendered completely inactive. This construct, which also will typically contain at least about 500 bases of DNA that correspond to the native (endogenous) 5' and 3' DNA sequences adjacent to the promoter segment that has been modified, may be introduced into the appropriate cells (either *ex vivo* or *in vivo*) either directly or via a viral vector as described herein. Typically, the integration of the construct into the genomic DNA of the cells will be via homologous recombination, where the 5' and 3' DNA sequences in the promoter construct can serve to help integrate the modified promoter region via hybridization to the endogenous chromosomal DNA.

Therapeutic Uses

CHL nucleic acid molecules, polypeptides, and agonists and antagonists thereof can be used to treat, diagnose, ameliorate, or prevent a number of diseases, disorders, or conditions, including those recited herein.

CHL polypeptide agonists and antagonists include those molecules which regulate CHL polypeptide activity and either increase or decrease at least one activity of the mature form of the CHL polypeptide. Agonists or antagonists may be co-factors, such as a protein, peptide, carbohydrate, lipid, or small molecular weight molecule, which interact with CHL polypeptide and thereby regulate its activity. Potential polypeptide agonists or antagonists include antibodies that

react with either soluble or membrane-bound forms of CHL polypeptides that comprise part or all of the extracellular domains of the said proteins. Molecules that regulate CHL polypeptide expression typically include nucleic acids encoding CHL polypeptide that can act as anti-sense regulators of expression.

5 Preliminary biological and biochemical characterization suggests several therapeutic utilities for CHL polypeptides. CHL polypeptides, fragments, variants, and/or derivatives may be used to prevent or treat bone diseases such as osteopetrosis and osteoporosis, aid in tissue regeneration and wound healing, or function in hematopoietic stem cell-genesis and expansion.

10 In clinical settings, the adult body is the major therapeutic target, and proteins that are produced and work in a normal healthy body may, therefore, give significant therapeutic benefits when they have been shown to function in organ homeostasis. In an adult organism, one of the major roles of the BMP-family of gene products – specifically BMP2 and BMP4 – is the regulation of bone-mass.
15 Since BMP1 has been shown to cleave and inactivate CHD and probably CHL polypeptide, and has been isolated with BMP2 and BMP3 from bone (Wozney *et al.*, 1988, *Science* 242:1528-34; Celeste *et al.*, 1990, *Proc. Nat. Acad. Sci. USA* 87: 9843-47), it is possible that CHL polypeptide and CHD play key regulatory roles in osteogenesis. This implies that one of the rate-limiting steps for the control of
20 bone mass may be the regulation of the BMP2/4 activity through CHL polypeptide and BMP1, as well as through Noggin. Thus, by administering CHL polypeptides or anti-CHL antibodies, the amount and activity of CHL polypeptide may be controlled and the bone density of in an adult decreased or increased as desired.

25 The direct delivery of BMP4 or other BMP-family members to the regenerating bone through the blood stream appears to be a straightforward therapeutic concept for treatment of osteopetrosis. However, it may be difficult to accomplish since BMP4 is known to travel only a short distance *in vivo* (Jones *et al.*, 1996, *Curr. Biol.* 6:1468-75). By forming a complex with CHL polypeptide,
30 BMP may migrate further – as in the case of CHD in an embryo leading to the formation of a concentration gradient for BMP4 (Jones and Smith, 1998, *Dev.*

Biol. 194:12-17). Patients suffering from either osteopetrosis or osteoporosis would benefit from improved treatments.

BMP polypeptides have been shown to function in organ formation during late embryogenesis. It is well-known that organ formation in embryonic kidney, lung, and gut are affected by BMP4 expression (Hogan 1996, *Genes Dev.* 10:1580-94). CHL polypeptide is shown herein to be expressed in the lung and small intestine, while CHD is known to be expressed in the kidney. Thus, it is possible that by using a combination of BMP4 and CHL polypeptide (or of BMP and CHD) the proliferation and differentiation of progenitor cells in these tissues could be controlled permitting for the regulation of tissue regeneration or wound healing *in vivo*.

Agonists or antagonists of CHL polypeptide function may be used (simultaneously or sequentially) in combination with one or more cytokines, growth factors, antibiotics, anti-inflammatories, and/or chemotherapeutic agents as is appropriate for the condition being treated.

Other diseases or disorders caused by or mediated by undesirable levels of CHL polypeptides are encompassed within the scope of the invention. Undesirable levels include excessive levels of CHL polypeptides and sub-normal levels of CHL polypeptides.

Uses of CHL Nucleic Acids and Polypeptides

Nucleic acid molecules of the invention (including those that do not themselves encode biologically active polypeptides) may be used to map the locations of the CHL gene and related genes on chromosomes. Mapping may be done by techniques known in the art, such as PCR amplification and *in situ* hybridization.

CHL nucleic acid molecules (including those that do not themselves encode biologically active polypeptides), may be useful as hybridization probes in diagnostic assays to test, either qualitatively or quantitatively, for the presence of a CHL nucleic acid molecule in mammalian tissue or bodily fluid samples.

Other methods may also be employed where it is desirable to inhibit the

activity of one or more CHL polypeptides. Such inhibition may be effected by nucleic acid molecules that are complementary to and hybridize to expression control sequences (triple helix formation) or to CHL mRNA. For example, antisense DNA or RNA molecules, which have a sequence that is complementary to at least a portion of a CHL gene can be introduced into the cell. Anti-sense probes may be designed by available techniques using the sequence of the CHL gene disclosed herein. Typically, each such antisense molecule will be complementary to the start site (5' end) of each selected CHL gene. When the antisense molecule then hybridizes to the corresponding CHL mRNA, translation of this mRNA is prevented or reduced. Anti-sense inhibitors provide information relating to the decrease or absence of a CHL polypeptide in a cell or organism.

Alternatively, gene therapy may be employed to create a dominant-negative inhibitor of one or more CHL polypeptides. In this situation, the DNA encoding a mutant polypeptide of each selected CHL polypeptide can be prepared and introduced into the cells of a patient using either viral or non-viral methods as described herein. Each such mutant is typically designed to compete with endogenous polypeptide in its biological role.

In addition, a CHL polypeptide, whether biologically active or not, may be used as an immunogen, that is, the polypeptide contains at least one epitope to which antibodies may be raised. Selective binding agents that bind to a CHL polypeptide (as described herein) may be used for *in vivo* and *in vitro* diagnostic purposes, including, but not limited to, use in labeled form to detect the presence of CHL polypeptide in a body fluid or cell sample. The antibodies may also be used to prevent, treat, or diagnose a number of diseases and disorders, including those recited herein. The antibodies may bind to a CHL polypeptide so as to diminish or block at least one activity characteristic of a CHL polypeptide, or may bind to a polypeptide to increase at least one activity characteristic of a CHL polypeptide (including by increasing the pharmacokinetics of the CHL polypeptide).

The CHL polypeptides of the present invention can be used to clone CHL polypeptide receptors, using an expression cloning strategy. Radiolabeled

(¹²⁵Iodine) CHL polypeptide or affinity/activity-tagged CHL polypeptide (such as an Fc fusion or an alkaline phosphatase fusion) can be used in binding assays to identify a cell type or cell line or tissue that expresses CHL polypeptide receptors. RNA isolated from such cells or tissues can be converted to cDNA, cloned into a mammalian expression vector, and transfected into mammalian cells (such as COS or 293 cells) to create an expression library. A radiolabeled or tagged CHL polypeptide can then be used as an affinity ligand to identify and isolate from this library the subset of cells that express the CHL polypeptide receptors on their surface. DNA can then be isolated from these cells and transfected into mammalian cells to create a secondary expression library in which the fraction of cells expressing CHL polypeptide receptors is many-fold higher than in the original library. This enrichment process can be repeated iteratively until a single recombinant clone containing a CHL polypeptide receptor is isolated. Isolation of the CHL polypeptide receptors is useful for identifying or developing novel agonists and antagonists of the CHL polypeptide signaling pathway. Such agonists and antagonists include soluble CHL polypeptide receptors, anti-CHL polypeptide receptor antibodies, small molecules, or antisense oligonucleotides, and they may be used for treating, preventing, or diagnosing one or more of the diseases or disorders described herein.

In addition to indicating that CHL polypeptides interact with and inhibit the activity of BMP4, preliminary biological and biochemical characterization suggests several other utilities for CHL polypeptides.

Since BMP2 is the most closely related BMP-family member to BMP4, CHL polypeptide may also inhibit the function of BMP2. In addition, interactions with other BMP-family members may also be detected. Furthermore, CHL polypeptide may also interact with a novel set of proteins that are not related to the BMP family. The murine, rat, and human CHL nucleic acids described herein are useful tools for obtaining the corresponding recombinant proteins. The recombinant CHL polypeptides of the present invention can be used to identify proteins that interact with this protein. For example, the CHL polypeptides may also be used to determine whether CHL polypeptide interacts directly with a

membrane or intracellular receptor.

The murine, rat, and human CHL nucleic acids of the present invention are also useful tools for isolating the corresponding chromosomal CHL polypeptide genes. For example, mouse chromosomal DNA containing CHL sequences can
5 be used to construct knockout mice, thereby permitting an examination of the *in vivo* role for CHL polypeptide. The human CHL genomic DNA can be used to identify heritable tissue-degenerating diseases.

As described herein, CHL mRNA has been identified in a set of bone marrow stroma cell lines that are known to support hematopoietic stem cells and
10 early progenitor cells, but has not been identified in stroma cell lines which support only committed progenitor cells (see Example 4). On the other hand, the CHL mRNA is detected in bone marrow, but not in fetal liver or in peripheral blood leukocytes. These observations imply that CHL polypeptide or CHL-interacting proteins might have some function in regulating hematopoietic stem
15 cell survival and maintenance, specifically in the bone marrow environment. The CHL polypeptides and CHL nucleic acids described herein may provide useful tools for *in vitro* expansion of hematopoietic stem cells. Alternatively, BMP4 or other putative CHL-interacting molecules can be used for regulating the survival and maintenance of hematopoietic stem cells.

20 BMP4 is an essential factor for generating hematopoietic progenitor cells from the mouse ES cells. However, the effective concentration of BMP4 falls into a narrow range (0.5 ng/ml to 5 ng/ml), which is consistent with the idea that the difference in the active concentration of BMP correlates with the difference in the resulting cell-type from the totipotent epiblast. A system for the reproducible
25 *in vitro* generation of hematopoietic stem cells from ES cells has not yet been disclosed. However, it may be achieved by precise control of the concentration of BMP4. The CHL polypeptides, anti-CHL antibodies, and CHL nucleic acids of the present invention would be useful tools for optimizing the culturing conditions for the *in vitro* generation of hematopoietic stem cells from ES cells.

30 Primitive hematopoietic stem cells have been recently defined in the mouse yolk sac (Yoder *et al.*, 1997, *Proc. Natl. Acad. Sci. USA* 94:6776-80). This

subclass of hematopoietic stem cells does not exhibit a marrow-repopulating activity in adults. However, when exposed to a newborn liver environment, the primitive stem cells are converted to long-term marrow-repopulating stem cells (*i.e.*, definitive stem cells). This can also be accomplished by culturing the primitive stem cells on certain stroma cell lines. The long-term maintenance of the definitive stem cells in culture and the long-term generation of the definitive stem cells from the primitive stem cells in culture have not yet been distinguished. The preferential expression of CHL mRNA in the stroma cell lines which support the definitive stem cell survival and maintenance suggests that interactions between BMP and CHL polypeptide or between BMP and CHD system might also function in the stem cell maintenance and/or generation. Thus the recombinant CHL polypeptides and CHL antibodies of the present invention may be useful tools for the both long-term maintenance and generation of the definitive hematopoietic stem cells *in vitro*. Alternatively, BMP4 or putative, novel CHL-interacting molecules can be used for controlling these processes.

The primitive hematopoietic stem cells have yet to be fully characterized. While primitive stem cells may be of a lymphocytic cell type, such cells may also be mesodermal precursors which are able to generate hematopoietic cell types as well as other mesodermal progeny. In support of this idea, adult bone marrow has recently been shown to contain endothelial progenitor cells, cells that regenerate liver (Petersen *et al.*, 1999, *Science* 284:1168-70), and a common stem cell that has a capability of deriving endothelial cells, muscle cells and hematopoietic cells *in vivo* (Ferrari *et al.*, 1998, *Science* 279:1528-30). Furthermore, the osteoblast cell lineage, which consists of the bone marrow stroma, is known to be derived from a mesenchymal stem cell that is present in bone marrow. The possibility that a common mesodermal stem cell is responsible for the generation of both stroma and hematopoietic cells has also been previously speculated.

Nevertheless, the fact that growth and differentiation of osteoblasts are regulated by BMP and that CHL polypeptide is expressed in bone marrow suggest that the BMP/CHL system might be involved in biological processes in which the bone marrow-mesodermal stem cells are involved. In this respect, the

recombinant CHL polypeptides and CHL antibodies of the present invention may be useful for characterization of such multi-lineage stem cells.

As described herein, primitive hematopoietic stem cells have been found in the mouse yolk sac. This class of hematopoietic stem cells has been shown to possess marrow-repopulating activity only following pre-exposure in a new-born liver environment. Since the presence of a primitive stem cell activity has never been investigated in the conventional hematopoietic sites of bone marrow, umbilical cord blood, and fetal liver, this suggests that there may be as yet unidentified (i.e. primitive) hematopoietic stem cells in these tissues. If the primitive hematopoietic stem cell is present in such hematopoietic tissues, it can be a novel target for *ex vivo* expansion, and could be a better target for gene transfer since such cells are more primitive than definitive stem cells. The concept that the conversion of primitive stem cells to definitive stem cells can also be achieved in culture on certain stroma cell lines makes this idea clinically feasible.

As discussed herein, through the use of BMP/CHD or BMP/CHL it may be possible to regulate primitive hematopoietic stem cells and thereby control adult-marrow repopulating stem cells. Alternatively, it may be possible to control stem cell genesis from a mesodermal stem cell. Thus, the CHL polypeptides and nucleic acids of the present invention, along with BMP, may be useful for *ex vivo* expansion of hematopoietic stem cells and gene therapy performed through such cells.

Deposits of cDNA encoding murine, rat, and human CHL polypeptide and human CHLd5 polypeptide, subcloned into pSPORT1 (Gibco BRL), having Accession Nos. PTA-961, PTA-962, PTA-963, or PTA-964, were made with the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 on November 16, 1999.

The following examples are intended for illustration purposes only, and should not be construed as limiting the scope of the invention in any way.

Example 1: Cloning of the Murine CHL Polypeptide Gene

Generally, materials and methods as described in Sambrook *et al. supra* were used to clone and analyze the gene encoding murine CHL polypeptide.

Sequences encoding murine CHL polypeptide were isolated from a normalized cDNA library of the mouse bone marrow stroma cell line, OP9, as a
5 sequence with statistically meaningful homology to the CR domains of chicken CHD. The normalized cDNA library was prepared by using the polymerase chain reaction (PCR) based normalization procedure of Takahashi and Ko, 1994, *Genomics* 23: 202-10.

Part of the normalized OP9 library of 3×10^5 independent clones was
10 screened for clones containing signal-like sequences using the amylase-based yeast signal trap method (U.S. Patent App. Serial No. 09/026,959). Individual colonies that gave a positive signal were picked, and the cDNA insert was amplified from each yeast clone by PCR using vector primers. The partial clone carrying the 5'-half of the coding region (clone tmsn1-00001-h4) was detected
15 among approximately 400 inserts sequenced.

A full-length cDNA clone (clone tmsn1-00001-h4-wze/agp-61975-a1) was then isolated from an OP9 full-length cDNA library constructed in the pcDNA3.1 expression vector (Invitrogen). Forty-eight individual sub-pools of the full-length cDNA library, each containing 2×10^4 independent clones, were screened using a
20 probe generated from the partial CHL nucleic acid sequence by PCR using the primers 2125-05 (5'-A-G-T-G-C-C-C-A-G-C-T-T-T-A-G-T-C-C-A-C-3'; SEQ ID NO: 20) and 2125-06 (5'-G-A-G-A-T-G-A-G-G-A-A-T-A-T-G-C-A-C-G-G-3'; SEQ ID NO: 21). The resulting 350 bp PCR fragment contained 267 bp of the CHL polypeptide coding region and 84 bp 5' non-coding sequence. Four positive
25 clones were obtained from a screen of 2×10^4 recombinant cDNA clones. The clone with the longest insert size (clone tmsn1-00001-h4-wze/agp-61975-a1) was sequenced.

Sequence analysis of the full-length cDNA for murine CHL polypeptide (clone 1-2) indicated that the gene comprises a 999 bp open reading frame
30 encoding a protein of 333 amino acids (Figures 1A-1C). The murine CHL

polypeptide sequence is predicted to contain a signal peptide (Figure 1A, predicted signal peptide indicated by underline).

Example 2: Cloning of the Rat CHL Polypeptide Gene

5 Generally, materials and methods as described in Sambrook *et al. supra* were used to clone and analyze the gene encoding rat CHL polypeptide.

Sequences encoding rat CHL polypeptide were isolated from a normalized rat prostate cDNA library as a result of expression-sequence-tag (EST) sequencing. A partial rat CHL nucleic acid sequence (clone srpb2-00279-a4) of
10 370 base pairs in length and encoding a polypeptide fragment that is 39% identical to CHD was identified through such analysis. The corresponding full-length rat CHL cDNA (clone srpb2-00279-a4-wz) was subsequently isolated by screening an oligo(dT)-primed rat prostate cDNA library using the partial rat CHL nucleic acid sequence as a probe.

15 Sequence analysis of the full-length cDNA for rat CHL polypeptide indicated that the gene comprises a 1146 bp open reading frame encoding a protein of 382 amino acids (Figures 2A-2D). The rat CHL polypeptide sequence is predicted to contain a signal peptide (Figure 2A, predicted signal peptide indicated by underline).

20

Example 3: Cloning of the Human CHL Polypeptide Gene

Generally, materials and methods as described in Sambrook *et al. supra* were used to clone and analyze the gene encoding human CHL polypeptide.

Sequences encoding human CHL polypeptide were isolated from a human
25 fetal brain cDNA library (Stratagene) using the partial rat CHL cDNA clone as a probe. The human CHL cDNA clone that was recovered lacked the amino-terminal end of the coding sequence, and the 5'-end part of the CHL mRNA was cloned separately using RACE methodology using a human heart marathon-ready cDNA kit (Clontech). The first round of PCR was performed using the 5'-primer
30 supplied with the kit (AP1) and the gene specific primer 2127-58 (5'-G-A-C-A-T-C-T-G-A-C-T-C-G-G-C-T-G-C-3'; SEQ ID NO: 22). The second PCR

amplification was performed using the 5'-primer supplied with the kit (AP2) and the gene specific primer 2212-48 (5'-T-C-A-C-G-C-A-G-T-A-A-A-C-C-A-A-C-3'; SEQ ID NO: 23).

5 The resulting PCR fragment was subcloned into the TOPO cloning vector (Invitrogen), the nucleotide sequence confirmed by sequencing, and the 5' human CHL fragment was then inserted into the partial human CHL cDNA described above in order to reconstruct the full-length human CHL cDNA (termed srpb2-00279-a4-wze).

10 Sequence analysis of the full-length cDNA for human CHL polypeptide indicated that the gene comprises a 1356 bp open reading frame encoding a protein of 452 amino acids (Figures 3A-3C). The human CHL polypeptide sequence is predicted to contain a signal peptide (Figure 3A, predicted signal peptide indicated by underlined and/or double-underlined).

15 A second form of human CHL cDNA, designated as CHLd5, was identified during the course of constructing the human CHL-FLAG fusion polypeptide (see Example 6). A full-length human CHLd5 DNA fragment in which the stop codon was replaced by a *Bam* HI site was obtained by PCR using heart cDNA (Clontech) as a template and the primers 2235-53 (5'-C-G-G-A-A-T-T-C-G-C-C-A-C-C-A-T-G-G-G-A-G-G-C-A-T-G-A-A-A-T-A-C-A-T-C-T-T-T-3'; SEQ ID NO: 24) and 2235-54 (5'-C-G-C-G-G-A-T-C-C-A-C-A-G-T-G-G-C-C-C-T-T-T-T-C-A-G-A-T-C-T-C-T-C-3'; SEQ ID NO: 25). The amplified PCR product was digested with *Eco* RI and *Bam* HI, gel purified, and then inserted into the pFLAG-CMV5a expression vector (Sigma). The resulting CHL-FLAG expression plasmid is designated as pFLAGhCHLd5. This form of human
20 CHL polypeptide has an internal deletion of 5 amino acids (Gly³¹⁹-Lys³²⁰-Lys³²¹-Ala³²²-Lys³²³) immediately following CR3. Interestingly, the identical peptide is present in the murine CHL polypeptide, but is lacking in the rat CHL polypeptide. Therefore, the rat CHL polypeptide may correspond to CHLd5.

30 Sequence analysis of the full-length cDNA for human CHLd5 polypeptide indicated that the gene comprises a 1341 bp open reading frame encoding a protein of 447 amino acids (Figures 4A-4C). The human CHLd5 polypeptide

sequence is predicted to contain a signal peptide (Figure 4A, predicted signal peptide indicated by underlined and/or double-underlined).

Computer analysis of the isolated murine, rat, and human sequences indicated that they possessed three repeated CR motifs, in contrast to the four repeats observed in CHD. Although CR motifs have been found in a number of other known proteins, including pro-collagen, a homology search in GenBank revealed that the CHL polypeptide motifs were most closely related to the CR motifs of CHD/SOG. The CR1 and CR3 of CHL polypeptide were particularly found to be highly homologous to the CR3 of CHD/SOD (indicated by gray boxes; Figure 5). The structural arrangement of the three CR motifs in CHL polypeptide seems to correspond to that of CR2-CR4 in CHD since CHL polypeptide lacks one CR motif as compared to CHD and also lacks the long gap between the CR1 and CR2 motifs of CHD. Figures 6A-6E illustrate the amino acid sequence alignment of human CHL polypeptide, human CHLd5 polypeptide, murine CHL polypeptide, rat CHL polypeptide, murine CHD, rat CHD, and human CHD. The murine CHL polypeptide is notable in that it is approximately one-third the size of the 948 amino acid murine CHD protein.

Example 4: CHL mRNA Expression

Multiple human or murine tissue northern blots (for mouse, mouse embryo, human, human II, and human immune system II, obtained from Clontech) were probed using a ^{32}P -dCTP-labeled 350 bp PCR fragment generated by amplification of the murine CHL cDNA clone (see Example 1).

The Northern blots were prehybridized in Express Hybridization solution (Clontech) for 1 hour at 68 °C and then were hybridized in the same solution with the addition of 1.3 ng/mL of labeled probe for 1 hour at 68 °C. Following hybridization, the filters were washed three times in 2X SSC and 0.05% SDS for 10 minutes at room temperature, and then twice in 0.1X SSC and 0.1% SDS for 20 minutes at 50 °C. Following washing, the blots were subjected to autoradiography.

Among the adult murine tissues analyzed, transcripts of 4 kb in length

having the highest expression were detected in the heart, brain, lung, and testis, transcripts with a moderate level of expression were detected in skeletal muscle, and transcripts with a lower level of expression were detected in the spleen, liver, and kidney (Figure 7A). Analysis of human tissue northern blots identified an abundant human transcript of 4-7 kb in length that cross reacts with the murine probe in the heart, brain, lung, placenta, prostate, small intestine and colon (mucosal lining). Lower levels of expression of this transcript were detected in liver, skeletal muscle, kidney, pancreas, thymus, lymph node, spleen, bone marrow, testis, and ovary (Figure 8). Expression below the level of detection on northern blots was found in peripheral blood leukocytes and fetal liver.

A comparative Northern analysis in mouse embryos provides additional support for a relationship between CHL polypeptide and CHD/SOG. While CHD/SOG has been shown to be expressed in E7 embryos – and at much lower levels in E11, E15 and E17 embryos (Pappano *et al.*, 1998, *Genomics* 52:236-39) – CHL RNA, in contrast, was found to be expressed in E11, E15, and E17 embryos but not in E7 embryos (Figure 7B). Furthermore, while CHL expression is detected in the adult heart and lung expression of CHD/SOG in these tissues is very weak (Pappano *et al.*, 1998 *Genomics* 52:236-39). In contrast, CHD/SOG expression is detected in the spleen, liver, and kidney (Pappano *et al.*, 1998, *Genomics* 52:236-39), while CHL expression in these tissues is very weak or below the level of detection. The expression pattern of CHL mRNA seems to contrast that of CHD/SOG with only a few exceptions (*i.e.*, brain and testis). Nevertheless, these results suggest that CHL polypeptide is not only structurally related to CHD/SOG but may also be functionally related.

Northern analyses were also performed on several bone-marrow-derived stroma cell lines (Figures 9A-9B). CHL polypeptide seems to be expressed differentially according to their properties. The stroma cell lines F10 and F4, which are known to support early hematopoietic progenitor cells (probably stem cells) in the formation of delayed cobblestone areas (CAs), were found to express CHL mRNA (Figure 9A). However, the stroma cell line D3 – a variant supporting only mature hematopoietic progenitor cells in the formation of short-

term CAs, does not express CHL mRNA. In this respect, CHL expression correlates with the ability of stroma cells to support survival, maintenance, and differentiation of hematopoietic stem cells. Since bone marrow, but not fetal liver, was found to express CHL mRNA (Figure 8), it would be expected that

5 CHL polypeptide is expressed by a stroma cell population specific to bone marrow rather than to fetal liver. The results of the Northern analysis also suggest that CHL polypeptide is probably not expressed in mature hematopoietic cell lineages, as peripheral blood leukocytes were found to not express appreciable levels of CHL mRNA (Figure 8).

10

The expression of CHL mRNA was localized by *in situ* hybridization. *In situ* hybridization was performed as described in Harlow and Lane, *Using Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1999), using antisense-RNAs synthesized with SP6 RNA polymerase (Ambion, Austin,

15 TX) from pSPmCHL5'. The pSPmCHL5' vector was prepared by removing the region between the Cla I and Not I sites of pSPORTmCHL and then linearizing the vector with Eco RI.

20

The expression of CHL mRNA was analyzed in those tissues determined to be CHL-expressing in the Northern blot analysis described above. Populations of fibroblast/connective tissue cells in the female reproductive tract, the gastrointestinal tract (Figure 10), and the outer medulla of the kidney (Figure 11) were found to express CHL mRNA. In the brain, CHL transcripts were localized in the cerebral cortex, the external plexiform layer of the olfactory bulb, and Purkinje cells of the cerebellum (Figure 12). The weak expression of CHL

25 mRNA detected in the lung and liver by Northern analysis, was actually localized to blood vessels supplying these tissues (Figure 10). Weak expression of CHL mRNA detected in the spleen and lymph nodes was localized to a non-lymphocytic cell population. Interestingly, *in situ* hybridization revealed additional sites of CHL mRNA expression. These additional sites include skin

30 mesenchyme and white adipose tissue. These observations suggest that, in the adult mouse, CHL mRNA is expressed mainly in cells of mesenchymal origin

(with the exception of neuronal cells in the brain).

In day 12.5 mouse embryos, a low level of signal was detected over cephalic mesenchyme in areas where the basioccipital and exoccipital bones will form, over mesenchymal cells in the lower jaw, and adjacent to the dorsal root ganglia in areas where vertebrae will form. A very low level of signal was also detected over the stomach wall (but not the epithelial lining of the stomach). In day 13.5 embryos, a signal was detected over the cartilage of the ribs, vertebra, limb bone (Figure 13), and trachea, and possibly over mesenchymal condensations destined to become tendon. In day 14.5 through 18.5 embryos, a detectable signal over the cartilage of the developing bones (Figure 13) was still present. In bones forming by endochondral formation, the signal appeared to be primarily over the hypertrophic chondrocytes in the growth regions, while the signal was more diffuse and less restricted in, for example, the bones of the head, which develop by intramembranous formation (Figure 12). Also at these stages, the pattern of expression in the gastrointestinal tract was similar to that of the adult, with a low level of signal being detected in a layer of fibroblast or smooth muscle cells between the mucosa and muscularis. One difference between embryonic and adult CHL mRNA expression concerned the esophagus where a signal was detected in the embryo but not the adult. This difference may have been due to the level of the embryonic sections that were examined.

At several embryonic stages, a signal was detected over relatively undifferentiated mesenchymal/connective tissue-type cells. For example, in day 14.5 embryos, CHL mRNA expression was detected in, and adjacent to, the salivary glands. In addition, CHL mRNA expression was detected in day 18.5 embryos in the subcutaneous tissue.

In summary, the strongest signal detected with the CHL mRNA probe was detected in the developing skeleton of the mouse embryo. Such expression was detected in the cartilage of bones undergoing either intramembranous or endochondral development. In the adult mouse CHL mRNA expression in bone or cartilage (as examined in the growth plate region of long bones, the articular cartilage of the joints, and tracheal cartilage) was either not present or was below

the level of sensitivity by *in situ* analysis. These observations support the argument that CHL mRNA may be an important regulator of bone and cartilage formation.

5 Example 5: Chromosomal Mapping of the Murine CHL Polypeptide Gene

Fluorescence *in situ* hybridization (FISH) analysis was used to determine the chromosomal localization of the murine CHL gene (Shi *et al.*, 1997, *Genomics* 45:42-47). A FISH probe was prepared from a BAC clone isolated from the Mouse ES-129/SvJ II BAC chromosome DNA library (Genome Systems) by PCR
10 using standard techniques and primers corresponding to the second CR domain of the murine CHL gene (5'-T-T-A-C-C-A-C-C-A-G-T-G-A-A-C-A-A-T-A-A-G-G-3'; SEQ ID NO: 26 and 5'-C-T-T-G-A-G-A-C-C-A-C-A-G-T-A-T-A-C-A-T-T-C-C-3'; SEQ ID NO: 27). The isolated BAC clone, F1038, was further examined by PCR using standard techniques and primers covering the 5' untranslated region
15 and signal peptide region of the murine CHL gene (5'-A-G-T-G-C-C-C-A-G-C-T-T-T-A-G-T-C-C-A-C-3'; SEQ ID NO: 28 and 5'-G-T-T-C-T-G-T-T-T-T-G-C-T-T-C-C-T-T-C-T-A-G-3'; SEQ ID NO: 29). From this analysis, it was concluded that the F1038 clone contains at least those exons of the murine CHL gene spanning from the 5' untranslated region to the second CR domain. The murine
20 CHL gene was mapped using F1038 DNA as a probe. It was found to be on the X chromosome.

A partial sequence of the human CHL gene, derived from the q22.1-23 region of the human X chromosome, was found in GenBank (Accession no. AL049176). In order to localize the murine CHL gene on the X chromosome, the
25 X centromere-specific P1 clone (#6856) was also used as a co-hybridization probe (Shi *et al.*, 1997, *Genomics* 45:42-47). A total of 80 metaphase cells were analyzed with 72 exhibiting specific labeling. Of these, 10 were used for co-hybridization experiments. The murine CHL gene was located at a position, which was 89% of the distance from the heterochromatic-euchromatic boundary
30 to the telomere of the X chromosome, an area corresponding to band XF3. Therefore, it has been concluded that both human and murine CHL genes are

located on a similar region of the X chromosome.

Example 6: Production of CHL Polypeptides

A. Expression of CHL Polypeptides in Bacteria

5 PCR is used to amplify template DNA sequences encoding a CHL polypeptide using primers corresponding to the 5' and 3' ends of the sequence. The amplified DNA products may be modified to contain restriction enzyme sites to allow for insertion into expression vectors. PCR products are gel purified and inserted into expression vectors using standard recombinant DNA methodology.

10 An exemplary vector, such as pAMG21 (ATCC no. 98113) containing the lux promoter and a gene encoding kanamycin resistance is digested with Bam HI and Nde I for directional cloning of inserted DNA. The ligated mixture is transformed into an *E. coli* host strain by electroporation and transformants are selected for kanamycin resistance. Plasmid DNA from selected colonies is isolated and

15 subjected to DNA sequencing to confirm the presence of the insert.

Transformed host cells are incubated in 2xYT medium containing 30 µg/mL kanamycin at 30°C prior to induction. Gene expression is induced by the addition of N-(3-oxohexanoyl)-dl-homoserine lactone to a final concentration of 30 ng/mL followed by incubation at either 30°C or 37°C for six hours. The

20 expression of CHL polypeptide is evaluated by centrifugation of the culture, resuspension and lysis of the bacterial pellets, and analysis of host cell proteins by SDS-polyacrylamide gel electrophoresis.

Inclusion bodies containing CHL polypeptide are purified as follows. Bacterial cells are pelleted by centrifugation and resuspended in water. The cell

25 suspension is lysed by sonication and pelleted by centrifugation at 195,000 xg for 5 to 10 minutes. The supernatant is discarded, and the pellet is washed and transferred to a homogenizer. The pellet is homogenized in 5 mL of a Percoll solution (75% liquid Percoll and 0.15 M NaCl) until uniformly suspended and then diluted and centrifuged at 21,600 xg for 30 minutes. Gradient fractions

30 containing the inclusion bodies are recovered and pooled. The isolated inclusion bodies are analyzed by SDS-PAGE.

A single band on an SDS polyacrylamide gel corresponding to *E. coli*-produced CHL polypeptide is excised from the gel, and the N-terminal amino acid sequence is determined essentially as described by Matsudaira *et al.*, 1987, *J. Biol. Chem.* 262:10-35.

5

B. Construction of CHL Polypeptide Mammalian Expression Vectors

A FLAG-tagged murine CHL polypeptide expression construct was prepared as follows. A full-length murine CHL DNA fragment, in which the stop codon was replaced by a *Bam*HI site, was obtained by PCR using the full-length
10 murine cDNA clone as a template and the primers 2149-76 (5'-G-C-T-A-G-C-G-G-C-C-G-C-G-C-C-A-C-C-A-T-G-G-A-T-G-G-C-A-T-G-A-A-A-T-A-C-A-T-C-A-T-T-T-C-3'; SEQ ID NO: 30) and 2149-77 (5'-G-G-T-A-C-C-G-G-A-T-C-C-A-C-C-A-A-A-G-G-C-A-G-G-G-C-C-T-C-C-A-G-C-3'; SEQ ID NO: 31).

The amplified PCR product was digested with *Not* I and *Bam* HI, gel
15 purified, and then inserted into the pFLAG-CMV-5a expression vector (Sigma) with the FLAG-sequence attached in-frame with the CHL sequence at its carboxyl-terminus. The fusion site between the CHL and FLAG sequences was subsequently confirmed by sequencing. The resulting CHL-FLAG expression plasmid is designated as pFLAGmCHL.

20 A FLAG-tagged murine CHD construct was prepared as follows. Murine CHD cDNA was isolated by RT-PCR from a mouse E7 embryo cDNA library (Clontech) using the primers 2170-06 (5'-G-C-T-A-G-C-G-G-C-C-G-C-G-C-C-A-C-C-A-T-G-C-C-G-A-G-C-C-T-C-C-C-G-G-C-C-C-C-G-3'; SEQ ID NO: 32) and 2170-07 (5'-G-G-A-T-C-C-G-T-C-G-A-C-G-G-A-G-T-G-C-T-C-C-G-C-T-T-C-T-T-T-C-T-C-C-A-G-3'; SEQ ID NO: 33).
25

The amplified PCR product was digested with *Not* I and *Sal* I, gel purified, and then inserted into the pFLAG-CMV-5a expression vector. Eleven clones were isolated and the corresponding cDNA inserts sequenced. To generate the final CHD-FLAG expression construct (labeled pFLAGmCHD), a 1.9 kb *Eco* RI
30 – *Bgl* II fragment of one of the identified clones (clone #16) was replaced with the corresponding 1.9 kb fragment of a separate clone (clone #3) to remove errors in

the sequence introduced as a result of PCR amplification.

To analyze the expression of either CHL-FLAG or CHD-FLAG, the pFLAGmCHL and pFLAGmCHD expression constructs were first introduced into human 293T cells using SuperFect transfection reagent (Qiagen), according to the manufacturer's suggested protocols. Prior to, and immediately following transfection, the 293T cells were maintained in DMEM supplemented with 10% Fetal Calf Serum (FCS, Hyclone). Following transfection, the cells were incubated overnight at 37 °C and 5% CO₂, and then the culture medium was renewed with serum-free IMDM, supplemented with 5-15% Knockout SR (Gibco BRL), and the cells were incubated for an additional 48 hours. The conditioned medium was then removed and CHL-FLAG and CHD-FLAG protein expression was analyzed by Western blot analysis using the anti-FLAG antibody M2 (Sigma). Approximately, 1 µg/mL of the CHD-FLAG and CHL-FLAG proteins were obtained.

To generate clones capable of stably expressing CHL-FLAG or CHD-FLAG, 293 cells were transfected using the calcium phosphate method with either linearized pFLAGmCHL or pFLAGmCHD and pGKneo (in a ratio of 30 to 1). After incubating the cells for 36 to 48 hours at 37 °C, the transfected cells were renewed with fresh medium containing 800 µg/mL G418 (Gibco BRL) for 12 days, and 12 clones were isolated for each transfection reaction. The expression levels of the CHL-FLAG and CHD-FLAG proteins were determined by Western blot analysis using the anti-FLAG M2 antibody. The clones with the highest expression levels (about 0.1 µg of CHL-FLAG or CHD-FLAG/mL of cell supernatant) were selected and expanded (Figure 14A).

CHL-FLAG and CHD-FLAG proteins were partially purified from the selected clones as follows. Clones expressing the proteins were expanded by growing the cells as suspension cells in a spinner culture with 293 SFM (Gibco BRL). Conditioned medium was collected and cell debris removed by centrifugation. CHL-FLAG and CHD-FLAG proteins were isolated by affinity chromatography using anti-FLAG M2 affinity gel (Sigma) packed in poly-prep chromatography columns (Bio-Rad). The bound fraction, containing the FLAG-

tagged CHL polypeptide or CHD protein was eluted by adding 100 µg/mL FLAG peptide (Sigma).

The human CHL-FLAG protein was also constructed in a similar way. A full-length human CHL DNA fragment in which the stop codon was replaced by a *Bam* HI site was obtained by PCR using the full-length human CHL cDNA clone as a template and the primers 2235-53 and 2235-54. The amplified PCR product was digested with *Eco* RI and *Bam* HI, gel purified, and then inserted into the pFLAG-CMV5a expression vector (Sigma). The resulting CHL-FLAG expression plasmid is designated as pFLAGhCHL. The pFLAGhCHL plasmid was introduced into human 293T cells as described, conditioned medium was removed, and the human CHL-FLAG expression was analyzed by Western blot analysis with anti-FLAG antibody M2 (Sigma) (Figure 14C).

C. Expression and Purification of CHL Polypeptide in Mammalian Cells

CHL polypeptide expression constructs are introduced into 293 EBNA or CHO cells using either a lipofection or calcium phosphate protocol.

To conduct functional studies on the CHL polypeptides that are produced, large quantities of conditioned media are generated from a pool of hygromycin selected 293 EBNA clones. The cells are cultured in 500 cm Nunc Triple Flasks to 80% confluence before switching to serum free media a week prior to harvesting the media. Conditioned media is harvested and frozen at -20°C until purification.

Conditioned media is purified by affinity chromatography as described below. The media is thawed and then passed through a 0.2 µm filter. A Protein G column is equilibrated with PBS at pH 7.0, and then loaded with the filtered media. The column is washed with PBS until the absorbance at A₂₈₀ reaches a baseline. CHL polypeptide is eluted from the column with 0.1 M Glycine-HCl at pH 2.7 and immediately neutralized with 1 M Tris-HCl at pH 8.5. Fractions containing CHL polypeptide are pooled, dialyzed in PBS, and stored at -70°C.

For Factor Xa cleavage of the human CHL polypeptide-Fc fusion polypeptide, affinity chromatography-purified protein is dialyzed in 50 mM Tris-

HCl, 100 mM NaCl, 2 mM CaCl₂ at pH 8.0. The restriction protease Factor Xa is added to the dialyzed protein at 1/100 (w/w) and the sample is digested overnight at room temperature.

5 Example 7: The N-terminal Amino Acid Sequence Determination of the Mature CHL-FLAG and CHD-FLAG Proteins

10 The affinity purified murine CHL-FLAG and murine CHD-FLAG proteins were separated from contaminated proteins by the SDS-polyacrylamide gel electrophoresis, and the major band was excised for amino acid sequence
15 determination with the gas-phase peptide sequencer, Procise 494 (Applied Biosystems). The results have indicated that the murine CHL-FLAG polypeptide precursor (SEQ ID NO: 34) is cleaved between Thr²² and Glu²³, and that the murine CHD-FLAG protein precursor (SEQ ID NO: 36) is cleaved between Gly²⁶ and Thr²⁷ to generate the mature forms of murine CHL-FLAG polypeptide (SEQ
ID NO: 35) and CHD-FLAG protein (SEQ ID NO: 37).

Example 8: Production of Anti-CHL Polypeptide Antibodies

20 Antibodies to CHL polypeptides may be obtained by immunization with purified protein or with CHL peptides produced by biological or chemical synthesis. Suitable procedures for generating antibodies include those described in Hudson and Bay, *Practical Immunology* (2nd ed., Blackwell Scientific Publications).

25 In one procedure for the production of antibodies, animals (typically mice or rabbits) are injected with a CHL antigen (such as a CHL polypeptide), and those with sufficient serum titer levels as determined by ELISA are selected for
30 hybridoma production. Spleens of immunized animals are collected and prepared as single cell suspensions from which splenocytes are recovered. The splenocytes are fused to mouse myeloma cells (such as Sp2/0-Ag14 cells), are first incubated in DMEM with 200 U/mL penicillin, 200 µg/mL streptomycin sulfate, and 4 mM glutamine, and are then incubated in HAT selection medium (hypoxanthine, aminopterin, and thymidine). After selection, the tissue culture supernatants are

taken from each fusion well and tested for anti-CHL antibody production by ELISA.

Alternative procedures for obtaining anti-CHL antibodies may also be employed, such as the immunization of transgenic mice harboring human Ig loci
5 for production of human antibodies, and the screening of synthetic antibody libraries, such as those generated by mutagenesis of an antibody variable domain.

Example 9: Biological Activity of Murine CHL in *Xenopus* Embryos

To assay the biological effect of native murine CHL polypeptide in
10 *Xenopus* embryos, a vector carrying the full-length murine CHL cDNA (pcDNA3mCHL), was used for *in vitro* RNA synthesis. The vector was first linearized with *Not* I and the capped mRNA was then transcribed with T7 RNA polymerase, using the mMESSAGE mMACHINE T7 kit (Ambion). For mCHL-FLAG, the *Eco* RI-*Sca* I fragment of FLAG-tagged CHL polypeptide was first
15 cloned into the *Eco* RI-*Not* I site of RN3 (Lemaire *et al.*, 1995, Cell 81:85-94), and then linearized with *Sfi* I. Then, capped mRNA was transcribed with T3 RNA polymerase. Following either synthesis, recovered RNA was subjected to two rounds of ethanol precipitation with 0.5 M ammonium acetate to remove unincorporated nucleotides, and then quantified by spectrophotometry at 260 nm.

20 *Xenopus* embryos were dejellied in 3% cysteine and staged as described in Nieuwkoop and Faber, *Normal Table of Xenopus laevis* (Daudin, ed., Garland Publishing, 1994). Embryos were placed into Steinberg's solution containing 5% Ficoll and 5 nL of RNA was injected into the two ventral blastomeres of 4-cell stage embryos. Following injection with various amounts of RNA, embryos were
25 cultured in 10% Steinberg's solution for 48 hours, and the embryos scored for ectopic axis. Of the embryos injected with 100 pg of either murine CHL-FLAG or native murine CHL RNA, all showed hyperdorsalization phenotypes, in which the anterior portions of the embryos are enlarged and the posterior parts are missing. This result is often observed when an excess amount of RNA carrying
30 axis duplication activity has been injected into the embryo. The injection of 30 pg RNA/embryo was found to be the optimal amount for generating an axis

duplication as a result of the introduction of the CHL polypeptide constructs (Figures 15A-15B). An axis duplication rate for CHL polypeptide of 86.7% (26/30 embryos) was observed whereas the rate for uninjected control embryos was 0% (0/31 embryos). The axis duplication rate for CHL-FLAG with poly-A was 78.9% (15/19 embryos), which is similar to that of the native CHL polypeptide without the FLAG-peptide and poly-A. As a positive control, experiments were also performed using the cloned murine CHD-FLAG (lacking poly-A). An axis duplication rate of 82.6% (19/23 embryos) was obtained using 1 ng of RNA. CHL polypeptide, with or without a FLAG-tag, is active in antagonizing the endogenous ventralizing factor (presumably, BMP4) in a similar fashion as CHD.

Example 10: Biological Activity of Murine CHL Polypeptide in ES Cells

The ability of CHL polypeptide to inhibit the activity of BMP4 was assayed in mouse embryonic stem (ES) cell culture as follows. E14 ES cells were maintained and differentiated as described by Nakayama *et al.*, 1998, *Blood* 91: 2283-95, with the exception that 0.9% methylcellulose (Stem Cell Technology) was added to the differentiation medium. Serum-free differentiation was achieved by replacing the FCS with Knockout-SR (Gibco BRL), at a concentration of 15%, at the pre-culture stage. The initial cell concentration was between 2500 and 4500 cells/ml. Rat SCF was added at 100 ng/ml to all of the differentiation cultures. Differentiated cells, aggregated as embryoid bodies, were collected, resuspended in 0.25% collagenase mix (a 1:1 mixture of Collagenase D (Boehringer Mannheim) and Collagenase XI (Sigma)) and 15% FCS in PBS, and then incubated for 60 minutes at 37 °C. A single cell suspension was then obtained by passing the cells through a 21-gauge needle followed by filtration through a 40-μ mesh. The cells were spun, resuspended in 0.5% BSA in PBSA at 5×10^6 cells/ml, and then stained with 2-20 μg/mL of antibodies against the hematopoietic cell markers CD34, Ter119, and CD45 (Pharmingen). The stained samples were analyzed on a FACScan (Becton Dickinson). Both Ter119+ erythroid cell formation and CD45+ macrophage cell generation were dependent

on the addition of between 0.5 to 2 ng/ml of human BMP4 protein (R&D Systems).

To assay the activity of the murine CHL-FLAG protein, a BMP4 concentration of 0.5 ng/mL was utilized, resulting in approximately a half-maximal level of Ter119+ erythroid cell formation during 7 days of differentiation. The CHL-FLAG protein made by the transient transfection of 293T cells was added at 0.2 to 2 mL/assay, corresponding to approximately 10 to 100 ng/mL of the CHL-FLAG protein/assay. Decreased levels of CD45+ and Ter119+ cells were detected depending upon the amount of CHL-FLAG protein that was added. For example, the percentage of Ter119+ cells was reduced by 35-50% when 50 to 100 ng/mL of CHL-FLAG protein was added (Figure 16). This suggests that CHL-FLAG protein is active, perhaps directly, in inhibiting BMP4 activity.

Example 11: Direct Interaction of CHL Polypeptide and BMP4

The direct interaction of *Xenopus* CHD protein with human BMP4 protein has been previously demonstrated (Piccolo *et al.*, 1996, *Cell* 86: 589-98). Similar experiments were performed using murine CHL polypeptide. An anti-FLAG antibody M2-conjugated agarose gel (Sigma), recognizing the carboxyl-terminus of the CHL-FLAG and CHD-FLAG fusion proteins was used to immunoprecipitate the CHL-BMP4 complex. Bound BMP4 protein was quantified by Western blot analysis using an anti-BMP2/4 goat polyclonal antibody (Santa Cruz).

Prior to immunoprecipitation, partially purified CHL-FLAG or CHD-FLAG and BMP4 were incubated in TBS (50 mM Tris HC mM NaCl, pH 7.4) for 1 hour at 4 °C. Following incubation of the proteins, anti-FLAG M2 affinity gel was added and the reaction incubated for an additional 2 hours at 4 °C in an Eppendorf mixer. After which, the agarose gels were spun down for 3 minutes and washed twice in 1 ml PBS. Following addition of gel loading buffer, the reactions were boiled for 10 minutes, electrophoresed through SDS-polyacrylamide gels under reducing conditions, and then electroblotted onto

nitrocellulose membranes.

The filter was subsequently blocked with TBST (10 mM Tris-HCl pH7.5, 0.9% NaCl, 0.1% Tween-20) containing 3% BSA and incubated in the same buffer with a 300-fold dilution of the anti-BMP2/4 antibody for 2 hours at room temperature. The blot was visualized using peroxidase-conjugated anti-goat secondary antibody (Pierce) and the chemiluminescent ECL kit (Amersham). The co-immunoprecipitation of BMP4 protein with either CHL-FLAG or CHD-FLAG suggested that there was a direct physical interaction between CHL polypeptide, or CHD, and BMP4 (Figure 14B).

Example 12: BMP4-Dependent Cell Proliferation and Survival

To analyze CHL polypeptide-mediated inhibition of BMP4-dependent proliferation and survival in A5-F stromal cells, 100 µl of test sample diluted in Iscove's modified Dulbecco medium (IMDM), 40 µl of 5X serum-free media containing bovine insulin (500 µg/ml), and 2000 A5-F murine bone marrow stromal cells (in a volume of 60 µl in IMDM) were added to each well in a 96-well mouse Collagen IV coated plate (Becton Dickinson). The culture was then incubated for 72 hours at 37°C and 5% CO₂. Following incubation, 22 µl of Alamar Blue Cell Proliferation Indicator (Biosource) was added to each well and the culture was incubated for an additional 24 hours. Following this incubation, the wells were read on a fluorescence microplate reader (BioTech Instruments FL500; using the following settings: excitation at 530/25 nm; emission at 590/35 nm; and sensitivity at 36). In this assay, Alamar Blue Cell Proliferation Indicator serves as a fluorometric growth indicator or detector of metabolic activity. Specifically, Alamar Blue Cell Proliferation Indicator acts as an oxidation-reduction indicator that fluoresces in response to the chemical reduction of growth medium resulting from cell growth. In the presence of continued cell growth, a reduced environment is maintained in the culture medium, while in the presence of inhibited cell growth, an oxidized environment is maintained. Reduction related to cell growth causes the redox indicator to change from an

oxidized form (*i.e.*, non-fluorescent, blue) to a reduced form (*i.e.*, fluorescent, red). Figures 17-18 illustrate the results of BMP-4 dependent cell proliferation and survival assays following incubation of A5-F cells in BMP-4 protein (Figure 17) or CHL polypeptide (Figure 18).

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Example 13: Expression of CHL Polypeptide in Transgenic Mice

To assess the biological activity of CHL polypeptide, a construct encoding a CHL polypeptide/Fc fusion protein under the control of a liver specific ApoE promoter is prepared. The delivery of this construct is expected to cause
10 pathological changes that are informative as to the function of CHL polypeptide. Similarly, a construct containing the full-length CHL polypeptide under the control of the beta actin promoter is prepared. The delivery of this construct is expected to result in ubiquitous expression.

To generate these constructs, PCR is used to amplify template DNA
15 sequences encoding a CHL polypeptide using primers that correspond to the 5' and 3' ends of the desired sequence and which incorporate restriction enzyme sites to permit insertion of the amplified product into an expression vector. Following amplification, PCR products are gel purified, digested with the appropriate restriction enzymes, and ligated into an expression vector using
20 standard recombinant DNA techniques. For example, amplified CHL polypeptide sequences can be cloned into an expression vector under the control of the human β -actin promoter as described by Graham *et al.*, 1997, *Nature Genetics*, 17:272-74 and Ray *et al.*, 1991, *Genes Dev.* 5:2265-73.

Following ligation, reaction mixtures are used to transform an *E. coli* host
25 strain by electroporation and transformants are selected for drug resistance. Plasmid DNA from selected colonies is isolated and subjected to DNA sequencing to confirm the presence of an appropriate insert and absence of mutation. The CHL polypeptide expression vector is purified through two rounds of CsCl density gradient centrifugation, cleaved with a suitable restriction
30 enzyme, and the linearized fragment containing the CHL polypeptide transgene is

purified by gel electrophoresis. The purified fragment is resuspended in 5 mM Tris, pH 7.4, and 0.2 mM EDTA at a concentration of 2 mg/mL.

Single-cell embryos from BDF1 x BDF1 bred mice are injected as described (PCT Pub. No. WO 97/23614). Embryos are cultured overnight in a CO₂ incubator and 15-20 two-cell embryos are transferred to the oviducts of a pseudopregnant CD1 female mice. Offspring obtained from the implantation of microinjected embryos are screened by PCR amplification of the integrated transgene in genomic DNA samples as follows. Ear pieces are digested in 20 mL ear buffer (20 mM Tris, pH 8.0, 10 mM EDTA, 0.5% SDS, and 500 mg/mL proteinase K) at 55°C overnight. The sample is then diluted with 200 mL of TE, and 2 mL of the ear sample is used in a PCR reaction using appropriate primers.

At 8 weeks of age, transgenic founder animals and control animals are sacrificed for necropsy and pathological analysis. Portions of spleen are removed and total cellular RNA isolated from the spleens using the Total RNA Extraction Kit (Qiagen) and transgene expression determined by RT-PCR. RNA recovered from spleens is converted to cDNA using the SuperScript™ Preamplification System (Gibco-BRL) as follows. A suitable primer, located in the expression vector sequence and 3' to the CHL polypeptide transgene, is used to prime cDNA synthesis from the transgene transcripts. Ten mg of total spleen RNA from transgenic founders and controls is incubated with 1 mM of primer for 10 minutes at 70°C and placed on ice. The reaction is then supplemented with 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 10 mM of each dNTP, 0.1 mM DTT, and 200 U of SuperScript II reverse transcriptase. Following incubation for 50 minutes at 42°C, the reaction is stopped by heating for 15 minutes at 72°C and digested with 2U of RNase H for 20 minutes at 37°C. Samples are then amplified by PCR using primers specific for CHL polypeptide.

Example 14: Biological Activity of CHL Polypeptide in Transgenic Mice

Prior to euthanasia, transgenic animals are weighed, anesthetized by isoflurane and blood drawn by cardiac puncture. The samples are subjected to hematology and serum chemistry analysis. Radiography is performed after

terminal exsanguination. Upon gross dissection, major visceral organs are subject to weight analysis.

Following gross dissection, tissues (*i.e.*, liver, spleen, pancreas, stomach, the entire gastrointestinal tract, kidney, reproductive organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, esophagus, thyroid, adrenals, urinary bladder, lymph nodes and skeletal muscle) are removed and fixed in 10% buffered Zn-Formalin for histological examination. After fixation, the tissues are processed into paraffin blocks, and 3 mm sections are obtained. All sections are stained with hematoxylin and eosin, and are then subjected to histological analysis.

The spleen, lymph node, and Peyer's patches of both the transgenic and the control mice are subjected to immunohistology analysis with B cell and T cell specific antibodies as follows. The formalin fixed paraffin embedded sections are deparaffinized and hydrated in deionized water. The sections are quenched with 3% hydrogen peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat monoclonal anti-mouse B220 and CD3 (Harlan, Indianapolis, IN). Antibody binding is detected by biotinylated rabbit anti-rat immunoglobulins and peroxidase conjugated streptavidin (BioGenex, San Ramon, CA) with DAB as a chromagen (BioTek, Santa Barbara, CA). Sections are counterstained with hematoxylin.

After necropsy, MLN and sections of spleen and thymus from transgenic animals and control littermates are removed. Single cell suspensions are prepared by gently grinding the tissues with the flat end of a syringe against the bottom of a 100 mm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells are washed twice, counted, and approximately 1×10^6 cells from each tissue are then incubated for 10 minutes with 0.5 μ g CD16/32(Fc γ III/II) Fc block in a 20 μ L volume. Samples are then stained for 30 minutes at 2-8°C in a 100 μ L volume of PBS (lacking Ca^+ and Mg^+), 0.1% bovine serum albumin, and 0.01% sodium azide with 0.5 μ g antibody of FITC or PE-conjugated monoclonal antibodies against CD90.2 (Thy-1.2), CD45R (B220), CD11b(Mac-1), Gr-1, CD4, or CD8

While the present invention has been described in terms of the preferred
5 embodiments, it is understood that variations and modifications will occur to
those skilled in the art. Therefore, it is intended that the appended claims cover
all such equivalent variations that come within the scope of the invention as
claimed.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

5 (a) the nucleotide sequence as set forth in any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11;

(b) the nucleotide sequence of the DNA insert in any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964;

10 (c) a nucleotide sequence encoding the polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(d) a nucleotide sequence which hybridizes under moderately or highly stringent conditions to the complement of any of (a) - (c); and

(e) a nucleotide sequence complementary to any of (a) - (c).

15 2. An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide which is at least about 70 percent identical to the polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, wherein the encoded
20 polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(b) a nucleotide sequence encoding an allelic variant or splice variant of the nucleotide sequence as set forth in any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, the nucleotide sequence of the DNA insert in
25 any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964, or (a);

(c) a region of the nucleotide sequence of any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, the DNA insert in any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964, (a), or (b) encoding a polypeptide fragment of at least about 25 amino acid residues, wherein the
30 polypeptide fragment has an activity of the encoded polypeptide as set forth in

any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or is antigenic;

(d) a region of the nucleotide sequence of any of SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 7, or SEQ ID NO: 11, the DNA insert in any of ATCC
5 Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964, or any of (a) - (c) comprising a fragment of at least about 16 nucleotides;

(e) a nucleotide sequence which hybridizes under moderately or highly stringent conditions to the complement of any of (a) - (d); and

(f) a nucleotide sequence complementary to any of (a) - (d).

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3. An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least
15 one conservative amino acid substitution, wherein the encoded polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(b) a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least
20 one amino acid insertion, wherein the encoded polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(c) a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least
25 one amino acid deletion, wherein the encoded polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(d) a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 which has a C-
30 and/or N- terminal truncation, wherein the encoded polypeptide has an activity of

the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(e) a nucleotide sequence encoding a polypeptide as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one modification selected from the group consisting of amino acid substitutions, amino acid insertions, amino acid deletions, C-terminal truncation, and N-terminal truncation, wherein the encoded polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(f) a nucleotide sequence of any of (a) - (e) comprising a fragment of at least about 16 nucleotides;

(g) a nucleotide sequence which hybridizes under moderately or highly stringent conditions to the complement of any of (a) - (f); and

(h) a nucleotide sequence complementary to any of (a) - (e).

4. A vector comprising the nucleic acid molecule of any of Claims 1, 2, or 3.

5. A host cell comprising the vector of Claim 4.

6. The host cell of Claim 5 that is a eukaryotic cell.

7. The host cell of Claim 5 that is a prokaryotic cell.

8. A process of producing a CHL polypeptide comprising culturing the host cell of Claim 5 under suitable conditions to express the polypeptide, and optionally isolating the polypeptide from the culture.

9. A polypeptide produced by the process of Claim 8.

10. The process of Claim 8, wherein the nucleic acid molecule comprises promoter DNA other than the promoter DNA for the native CHL polypeptide operatively linked to the DNA encoding the CHL polypeptide.

11. The isolated nucleic acid molecule according to Claim 2, wherein the percent identity is determined using a computer program selected from the group consisting of GAP, BLASTN, FASTA, BLASTA, BLASTX, BestFit, and the Smith-Waterman algorithm.

12. A process for determining whether a compound inhibits CHL polypeptide activity or CHL polypeptide production comprising exposing a cell according to any of Claims 5, 6, or 7 to the compound and measuring CHL polypeptide activity or CHL polypeptide production in said cell.

13. An isolated polypeptide comprising the amino acid sequence selected from the group consisting of:

(a) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12; and

(b) the amino acid sequence encoded by the DNA insert in any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964.

14. An isolated polypeptide comprising the amino acid sequence selected from the group consisting of:

(a) the amino acid sequence as set forth in any of SEQ ID NO: 3, SEQ ID NO: 6, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 13, or SEQ ID NO: 14, optionally further comprising an amino-terminal methionine;

(b) an amino acid sequence for an ortholog of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(c) an amino acid sequence which is at least about 70 percent identical to the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, wherein the polypeptide has an activity of the polypeptide

set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(d) a fragment of the amino acid sequence set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 comprising at least about 25 amino acid residues, wherein the fragment has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or is antigenic; and

(e) an amino acid sequence for an allelic variant or splice variant of the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, the amino acid sequence encoded by the DNA insert in any of ATCC Deposit Nos. PTA-961, PTA-962, PTA-963, or PTA-964, or any of (a) - (c).

15. An isolated polypeptide comprising the amino acid sequence selected from the group consisting of:

(a) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one conservative amino acid substitution, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(b) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one amino acid insertion, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(c) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one amino acid deletion, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12;

(d) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 which has a C- and/or N- terminal

truncation, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12; and

- (e) the amino acid sequence as set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12 with at least one modification
5 selected from the group consisting of amino acid substitutions, amino acid insertions, amino acid deletions, C-terminal truncation, and N-terminal truncation, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

- 10 16. An isolated polypeptide encoded by the nucleic acid molecule of any of Claims 1, 2, or 3, wherein the polypeptide has an activity of the polypeptide set forth in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

- 15 17. The isolated polypeptide according to Claim 14, wherein the percent identity is determined using a computer program selected from the group consisting of GAP, BLASTP, FASTA, BLASTA, BLASTX, BestFit, and the Smith-Waterman algorithm.

- 20 18. A selective binding agent or fragment thereof which specifically binds the polypeptide of any of Claims 13, 14, or 15.

19. The selective binding agent or fragment thereof of Claim 18 that specifically binds the polypeptide comprising the amino acid sequence as set forth
25 in any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12, or a fragment thereof.

20. The selective binding agent of Claim 18 that is an antibody or fragment thereof.

21. The selective binding agent of Claim 18 that is a humanized antibody.

22. The selective binding agent of Claim 18 that is a human antibody
5 or fragment thereof.

23. The selective binding agent of Claim 18 that is a polyclonal antibody or fragment thereof.

10 24. The selective binding agent Claim 18 that is a monoclonal antibody or fragment thereof.

25. The selective binding agent of Claim 18 that is a chimeric antibody
or fragment thereof.

15 26. The selective binding agent of Claim 18 that is a CDR-grafted antibody or fragment thereof.

20 27. The selective binding agent of Claim 18 that is an antiidiotypic antibody or fragment thereof.

28. The selective binding agent of Claim 18 that is a variable region fragment.

25 29. The variable region fragment of Claim 28 that is a Fab or a Fab' fragment.

30 30. A selective binding agent or fragment thereof comprising at least one complementarity determining region with specificity for a polypeptide having the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.

31. The selective binding agent of Claim 18 that is bound to a detectable label.

5 32. The selective binding agent of Claim 18 that antagonizes CHL polypeptide biological activity.

33. A method for treating, preventing, or ameliorating a CHL polypeptide-related disease, condition, or disorder comprising administering to a
10 patient an effective amount of a selective binding agent according to Claim 18.

34. A selective binding agent produced by immunizing an animal with a polypeptide comprising an amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, or SEQ ID NO: 12.
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35. A hybridoma which produces a selective binding agent which is capable of binding a polypeptide according to any of Claims 1, 2, or 3.

36. A method of detecting or quantitating the amount of CHL polypeptide using the anti-CHL antibody or fragment of Claim 18.
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37. A composition comprising the polypeptide of any of Claims 13, 14, or 15, and a pharmaceutically acceptable formulation agent.

25 38. The composition of Claim 37, wherein the pharmaceutically acceptable formulation agent is a carrier, adjuvant, solubilizer, stabilizer, or anti-oxidant.

39. The composition of Claim 37, wherein the polypeptide comprises the amino acid sequence as set forth in any of SEQ ID NO: 3, SEQ ID NO: 6, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 13, or SEQ ID NO: 14.
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40. A polypeptide comprising a derivative of the polypeptide of any of Claims 13, 14, or 15.

5 41. The polypeptide of Claim 40 that is covalently modified with a water-soluble polymer.

42. The polypeptide of Claim 41, wherein the water-soluble polymer is selected from the group consisting of polyethylene glycol, monomethoxy-
10 polyethylene glycol, dextran, cellulose, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide copolymers, polyoxyethylated polyols, and polyvinyl alcohol.

43. A composition comprising a nucleic acid molecule of any of
15 Claims 1, 2, or 3 and a pharmaceutically acceptable formulation agent.

44. The composition of Claim 43, wherein said nucleic acid molecule is contained in a viral vector.

20 45. A viral vector comprising a nucleic acid molecule of any of Claims 1, 2, or 3.

46. A fusion polypeptide comprising the polypeptide of any of Claims 13, 14, or 15 fused to a heterologous amino acid sequence.

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47. The fusion polypeptide of Claim 46, wherein the heterologous amino acid sequence is an IgG constant domain or fragment thereof.

48. A method for treating, preventing, or ameliorating a medical
30 condition comprising administering to a patient the polypeptide of any of Claims

13, 14, or 15, or the polypeptide encoded by the nucleic acid of any of Claims 1, 2, or 3.

49. The method of Claim 48, wherein the medical condition being
5 treated, prevented, or ameliorated is osteopetrosis or osteoporosis.

50. A method of diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising:

(a) determining the presence or amount of expression of the
10 polypeptide of any of Claims 13, 14, or 15, or the polypeptide encoded by the nucleic acid molecule of any of Claims 1, 2, or 3 in a sample; and

(b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or amount of expression of the polypeptide.
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51. A device, comprising:

(a) a membrane suitable for implantation; and

(b) cells encapsulated within said membrane, wherein said cells secrete a protein of any of Claims 13, 14, or 15; and
20 said membrane is permeable to said protein and impermeable to materials detrimental to said cells.

52. A method of identifying a compound which binds to a CHL polypeptide comprising:

(a) contacting the polypeptide of any of Claims 13, 14, or 15 with a compound; and
25

(b) determining the extent of binding of the CHL polypeptide to the compound.

30 53. The method of Claim 52, further comprising determining the activity of the polypeptide when bound to the compound.

54. A method of modulating levels of a polypeptide in an animal comprising administering to the animal the nucleic acid molecule of any of Claims 1, 2, or 3.

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55. A transgenic non-human mammal comprising the nucleic acid molecule of any of Claims 1, 2, or 3.

56. A process for determining whether a compound inhibits CHL polypeptide activity or CHL polypeptide production comprising exposing a transgenic mammal according to Claim 55 to the compound, and measuring CHL polypeptide activity or CHL polypeptide production in said mammal.

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ABSTRACT

The present invention provides Chordin-Like (CHL) polypeptides and nucleic acid molecules encoding the same. The invention also provides selective binding agents, vectors, host cells, and methods for producing CHL polypeptides.

- 5 The invention further provides pharmaceutical compositions and methods for the diagnosis, treatment, amelioration, and/or prevention of diseases, disorders, and conditions associated with CHL polypeptides.

FIG. 1A

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ccacgcgtcc ggagcgcccc agggagctca gagcttgtgc aagcgtggca gcaggaggag 60
gccagtcccc agcttttagtc caccgctcct ctccttgagg cccctgaatt gcatttttgc 120
gtagctcgaa ggagaaaaaa gtagaag atg gat ggc atg aaa tac atc att tcc 174
      Met Asp Gly Met Lys Tyr Ile Ile Ser
      1               5

tta ttt ttc atc ttt gtt ttc cta gaa gga agc aaa aca gaa caa gta 222
Leu Phe Phe Ile Phe Val Phe Leu Glu Gly Ser Lys Thr Glu Gln Val
10               15               20               25

aaa cac tca gac aca tat tgc gtg ttt caa gac aag aag tat aga gtg 270
Lys His Ser Asp Thr Tyr Cys Val Phe Gln Asp Lys Lys Tyr Arg Val
30               35               40

ggg gag aaa tgg cat ccc tac ctg gaa ccg tat gga ctg gtt tac tgt 318
Gly Glu Lys Trp His Pro Tyr Leu Glu Pro Tyr Gly Leu Val Tyr Cys
45               50               55

gtg aac tgc atc tgc tct gag aat ggg aat gtg ctt tgc agc cga gtc 366
Val Asn Cys Ile Cys Ser Glu Asn Gly Asn Val Leu Cys Ser Arg Val
60               65               70

aga tgt cca agt ctt cat tgc ctt tca ccc gtg cat att cct cat ctc 414
Arg Cys Pro Ser Leu His Cys Leu Ser Pro Val His Ile Pro His Leu
75               80               85

tgt tgc ccc cgc tgc cca gac tcc tta cca cca gtg aac aat aag gtg 462
Cys Cys Pro Arg Cys Pro Asp Ser Leu Pro Pro Val Asn Asn Lys Val
90               95               100               105

acc agc aag tca tgc gaa tac aat gga acc act tac caa cat gga gaa 510
Thr Ser Lys Ser Cys Glu Tyr Asn Gly Thr Thr Tyr Gln His Gly Glu
110               115               120

ctg ttc ata gct gaa ggg ctc ttt cag aac cgg caa ccc aat cag tgc 558
Leu Phe Ile Ala Glu Gly Leu Phe Gln Asn Arg Gln Pro Asn Gln Cys
125               130               135

agt cag tgt agc tgc tcg gag ggg aat gta tac tgt ggt ctc aag act 606
Ser Gln Cys Ser Cys Ser Glu Gly Asn Val Tyr Cys Gly Leu Lys Thr
140               145               150

tgc ccc aaa ctg acc tgt gca ttc cca gtc tct gtt cca gat tct tgc 654
Cys Pro Lys Leu Thr Cys Ala Phe Pro Val Ser Val Pro Asp Ser Cys
155               160               165

tgc cga gta tgc aga ggg gat gca gaa tta tcg tgg gaa cat gcg gat 702
Cys Arg Val Cys Arg Gly Asp Ala Glu Leu Ser Trp Glu His Ala Asp
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FIG. 1B

ggt gat atc ttc cgg caa cct gcc aac aga gaa gca aga cat tct tac 750
 Gly Asp Ile Phe Arg Gln Pro Ala Asn Arg Glu Ala Arg His Ser Tyr
 190 195 200

 ctc cgt tcc ccc tac gat cct cca cca aac aga caa gct gga ggt ctt 798
 Leu Arg Ser Pro Tyr Asp Pro Pro Asn Arg Gln Ala Gly Gly Leu
 205 210 215

 ccc cgc ttt cct ggg agc aga agt cac cgg gga gct gtt ata gat tcc 846
 Pro Arg Phe Pro Gly Ser Arg Ser His Arg Gly Ala Val Ile Asp Ser
 220 225 230

 cag caa gca tcc ggg acc atc gtg cag att gtc atc aat aac aag cac 894
 Gln Gln Ala Ser Gly Thr Ile Val Gln Ile Val Ile Asn Asn Lys His
 235 240 245

 aaa cat gga caa gtg tgt gtt tcc aat gga aag acc tac tct cat gga 942
 Lys His Gly Gln Val Cys Val Ser Asn Gly Lys Thr Tyr Ser His Gly
 250 255 260 265

 gag tcc tgg cac cca aat cta cga gca ttt ggc att gtg gaa tgt gta 990
 Glu Ser Trp His Pro Asn Leu Arg Ala Phe Gly Ile Val Glu Cys Val
 270 275 280

 cta tgc act tgt aat gtc acc aag caa gaa tgt aag aaa atc cac tgc 1038
 Leu Cys Thr Cys Asn Val Thr Lys Gln Glu Cys Lys Lys Ile His Cys
 285 290 295

 ccc aat cga tac ccc tgc aag tat cct caa aaa ata gat gga aag tgc 1086
 Pro Asn Arg Tyr Pro Cys Lys Tyr Pro Gln Lys Ile Asp Gly Lys Cys
 300 305 310

 tgc aag gtg tgc cca ggt aaa aag gca aaa ggt gca ttg gct gga ggc 1134
 Cys Lys Val Cys Pro Gly Lys Lys Ala Lys Gly Ala Leu Ala Gly Gly
 315 320 325

 cct gcc ttt ggt tgaatgagat tcacacatag tcctattcag tcttctttgt 1186
 Pro Ala Phe Gly
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 tcataaaaac tataaatgac ctgtcttata gttctaacga taatagttct agcaagaatg 1246
 aacttcatcc tttcgtcttc tgagacactg atggttgctt tgaaggaatt aactactcag 1306
 agtttctttt gtctacaatg tcaaacacat gccaaagtgc ttatcttggt cttgcttttc 1366
 taaattagag agtttacggt atcactgttt tagaaaaagt cacacctttc atggtttaaa 1426
 tcaccaactc acttcaagac ataatccagt actctttttca gatgagatat aaatgagtta 1486
 cagtggagag aaattagatt ctgatccaaa tgcattcaaat ccacaagtat cttaccccat 1546
 gtgaacattt taaagtttat tactgtgttc cacattgcta ttttaatttg caatttcttt 1606

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FIG. 1C

ttaaattttc tgagatattg tatctgtata tacttatggg gtacagtatg ttaattcaat 1666
acaaatatac aaggtataat tgtcaaata gggtaattat cattctctct cctctgattt 1726
tatccctaga ctcttctagt cattttaaaa tttatcatca attgggtttt tgatatggta 1786
actccactgt gctaaagaaa ccattcattc taatggcatt ttaggatcta ctatctaacc 1846
tctatctccc cttctgtt 1864

FIG. 2A

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ccacgcgtcc gagcgcccca gggagctcag agcgtgtgca agcgtggcag aaggaagagg 60
ccagtgccca gctttagccc accagtccta ggagtctctg agctgcattt tgcagtagct 120
caaaggagaa gagagtggaa a atg gaa ggc ata aaa tat atc gcc tcc ttg 171
                        Met Glu Gly Ile Lys Tyr Ile Ala Ser Leu
                        1         5         10
gtt ttc ttc ttt gtt ttc ctg gaa gca agc aaa aca gag cca gta aaa 219
Val Phe Phe Phe Val Phe Leu Glu Ala Ser Lys Thr Glu Pro Val Lys
                        15         20         25
cac tca gag aca tat tgc atg ttt caa gac aag aag tat aga gtt ggt 267
His Ser Glu Thr Tyr Cys Met Phe Gln Asp Lys Lys Tyr Arg Val Gly
                        30         35         40
gag aaa tgg cat ccc tac ctg gaa cca tat gga ctg gtt tac tgt gtg 315
Glu Lys Trp His Pro Tyr Leu Glu Pro Tyr Gly Leu Val Tyr Cys Val
                        45         50         55
aac tgc atc tgc tca gag aat ggg aat gtg ctt tgc agc cga gtc aga 363
Asn Cys Ile Cys Ser Glu Asn Gly Asn Val Leu Cys Ser Arg Val Arg
                        60         65         70
tgt cca act ctt cat tgc ctt tca ccc gtg cat att cct cat ctg tgt 411
Cys Pro Thr Leu His Cys Leu Ser Pro Val His Ile Pro His Leu Cys
                        75         80         85         90
tgc ccc cgt tgc cca gac tcc tta cca ccg atg aac aat aag gtg acc 459
Cys Pro Arg Cys Pro Asp Ser Leu Pro Pro Met Asn Asn Lys Val Thr
                        95         100         105
agc aag tcc tgc gaa tac aat ggg acc acc tac caa cac gga gag ctc 507
Ser Lys Ser Cys Glu Tyr Asn Gly Thr Thr Tyr Gln His Gly Glu Leu
                        110         115         120
ttc ata gct gaa ggg ctc ttt cag aac cgg cag ccc aat cag tgc agt 555
Phe Ile Ala Glu Gly Leu Phe Gln Asn Arg Gln Pro Asn Gln Cys Ser
                        125         130         135
cag tgc agc tgc tgc gag ggg aat gtg tat tgt ggt ctc aag act tgc 603
Gln Cys Ser Cys Ser Glu Gly Asn Val Tyr Cys Gly Leu Lys Thr Cys
                        140         145         150
ccc aaa ctg acc tgt gca ttc cca gtc tct gtt cca gat tcc tgc tgc 651
Pro Lys Leu Thr Cys Ala Phe Pro Val Ser Val Pro Asp Ser Cys Cys
                        155         160         165         170
cga gta tgc aga ggg gat gga gaa tta tca tgg gaa cat tct gat gct 699
Arg Val Cys Arg Gly Asp Gly Glu Leu Ser Trp Glu His Ser Asp Ala
                        175         180         185

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FIG. 2B

gat atc ttc cgg caa cct gcc aac aga gaa gca aga cat tct tac ctc	747
Asp Ile Phe Arg Gln Pro Ala Asn Arg Glu Ala Arg His Ser Tyr Leu	
190 195 200	
cggtcccccgtacgatcctccaagcagacaa gct gga ggt ctt cct	795
Arg Ser Pro Tyr Asp Pro Pro Ser Arg Gln Ala Gly Gly Leu Pro	
205 210 215	
cgc ttt gct ggg agc aga agt cac cgg gga gct gtc att gat tct cag	843
Arg Phe Ala Gly Ser Arg Ser His Arg Gly Ala Val Ile Asp Ser Gln	
220 225 230	
caa gca tca ggg acc atc gtg cag atc gtc atc aat aac aag cac aaa	891
Gln Ala Ser Gly Thr Ile Val Gln Ile Val Ile Asn Asn Lys His Lys	
235 240 245 250	
cat gga caa gtg tgt gtt tcc aat gga aag acc tat tct cac gga gaa	939
His Gly Gln Val Cys Val Ser Asn Gly Lys Thr Tyr Ser His Gly Glu	
255 260 265	
tcc tgg cat tca aat cta cga gct ttt ggc att gtg gaa tgt gtt cta	987
Ser Trp His Ser Asn Leu Arg Ala Phe Gly Ile Val Glu Cys Val Leu	
270 275 280	
tgc act tgt aat gtc acc aag caa gaa tgt aag aaa atc cac tgc ccc	1035
Cys Thr Cys Asn Val Thr Lys Gln Glu Cys Lys Lys Ile His Cys Pro	
285 290 295	
aat cga tac ccc tgc aag tat cct caa aaa tta gat gga aag tgc tgc	1083
Asn Arg Tyr Pro Cys Lys Tyr Pro Gln Lys Leu Asp Gly Lys Cys Cys	
300 305 310	
aag gtg tgc cca gaa gaa cct cca agt caa aac ttt gac agc aaa ggt	1131
Lys Val Cys Pro Glu Glu Pro Pro Ser Gln Asn Phe Asp Ser Lys Gly	
315 320 325 330	
tcc ttt tgt gga gaa gaa acc atg cct gta tat gag gct gtg ctc gtg	1179
Ser Phe Cys Gly Glu Glu Thr Met Pro Val Tyr Glu Ala Val Leu Val	
335 340 345	
gag gat gga gag aca gcc aga aaa gta gca ctg gag acc gag aaa cca	1227
Glu Asp Gly Glu Thr Ala Arg Lys Val Ala Leu Glu Thr Glu Lys Pro	
350 355 360	
cct caa gta gta ggt tca cgt ttg gac tat tcg aaa ggg cat tct cca	1275
Pro Gln Val Val Gly Ser Arg Leu Asp Tyr Ser Lys Gly His Ser Pro	
365 370 375	
gca ctt cca cat tgagaagatt tccaaggaga tgtttgggga gctccatcat	1327
Ala Leu Pro His	
380	
ttcaagctgg tgactcgaac caccatgaac cagtggaaga tcttcgctga aggagaagct	1387

FIG. 2C

cagctcagcc agatgtgctc aagtcgggtg tgcagaacag aactggaaga tttgggtccag 1447
 gttttgtacc tggagagacc tgaaaaggac cactgttaga caaaacagtc aggattgaat 1507
 agtatcaatc aaggaaaccc aagctgcagc tggactgccg gcttacttta ctttaagtcaa 1567
 cagtgtcca aaaccccaaa gtcaacctca gtcaaattat ccagtcacag cacaccttgt 1627
 tcctctatgt gcagcgggtg gccagccctc aaacatctcc tgtaaagaga atagaggagt 1687
 ctttaatggg ttctgggggt ggggggagaa gggataggac tttgtggtac agctctat 1747
 tctctgagaa tcacatttat ttgcaggtta aagtagaaaa gaaaaccact ttttagggat 1807
 tctatgtaga aagtcacaag agagagagag agagaaattg ctgagtttga gttggatcat 1867
 gccaaacaaa tttgtgtgaa atactttttg aatgttcaag tgtcttcctt actttaaaaa 1927
 tgttattcag ttgggtggtt aacagtcagg tgattatgga gcacatacct ataatatgtg 1987
 gagacctggg ttctagtctc agaactgaca aaaaaatttc tctcctcata tctcacatgc 2047
 acacacacac acacacacac acacacacac acacacacac acacacacac acagcacacg 2107
 aaactgcatt tctttctggc tcctaaacac ctttgtggtt gttcgtatcc agggaaacaa 2167
 actaaaaatg tatgcaaaaa actctgccct caagcctttg aggcagggtg taagaaatca 2227
 gccatagtct tagagtgaag aatgccattt gtgggtcttg tttccttcga agtactaaat 2287
 acattttgcc tagtaatatc acttctcttt tcttatctgg caccctcatt aggaaggtag 2347
 aatttgagaa actcatcaga aactaaattt attccaaaca aaatgacaat agaagaatat 2407
 aactgataaa aaataaaaata gtccattttt tgttttggtt ttacagctat aaatctaact 2467
 gattaatagg ctaatgatgc tcactaattt tcttgaggca atagtcacct aggcagacac 2527
 tttagggtga cacttttatt ctaaaagcct ttttaagggt aatttcctac tttgattaca 2587
 ggagttgaaa tgtaactttt caaaaaggct caatccttac aagcttctca acatcagttc 2647
 ttctgttaag tgctactgtt cattcacaga gctgagaatt ctggcaaaga tctttgtccc 2707
 aacccttcct aatatccttg ccttattctt gagcatgggt tgcagcaggg attgtgacag 2767
 cactacttct aaaatgttca tttgcagccc agtgccctca catcaatttt ccttcctgag 2827
 gcttggtttt agaaatcacc ttttgaaaaa actataacta ttccctagca aagatcatag 2887
 gttcactgga tctgtccatc tgccgagcat gaatgaactc acatgagtac taagaaatgt 2947
 gaagatcaag aaattctata tttccactc taagtgagaa aacatgatag gaaaaagtat 3007

FIG. 2D

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gaagagtctg gtctttacta gaacctgaca gagaagggaa ggctttgggg ccagggttc 3067
atgagacaaa cttcctgcc gccaattaca cattctcca agaagagaag catagggcgt 3127
cctgggctgc aaagacactg aacattattg aagatgtgat ggggcaatgc caaccctctg 3187
ctgcttcctt cttggaggaa acactatttc cagagtgcgg agatcaatca caggctctga 3247
aggaaagtgg tgattcctgt gctagacgat tcacccgcag ggaaggtggt gattcccgtg 3307
ctagatgatt cactcacaaa cttcccgcc cagggtgttct ctgaaagctt agcctcaagg 3367
gaacacctaa agagctcccc tacctacata aacccctgcc tccaagtgtg ggaactcacc 3427
tttctaaagc gctgtgggaa gcaggaactg ggcatctgtg ctaagtcaat gtagaatttc 3487
tccagcgttt taatgctggg tagaatatag agcatagggg aaagggggcca aactgcctat 3547
agttagtaga gaaaaatgaa tgtggttctt ttgtgcattt atgtgtatca taaacacttg 3607
ggaaagcaaa aaccataagc accattttgc aactttatcc attttccagt tagctcatgt 3667
aaacgagcac gaataacaaa acagtattac tctttcgac ttctcacagg acatgtaccc 3727
aaatacggta cttatttatg tagtcactgt gtttcaggac ttttacgtta ataaaatttt 3787
tattttaaatt tttaaaaaaa aaaaaaaaaa aaaaaaaaaa 3827

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Variable	Mean	SD	Min	Max	Skewness	Kurtosis	Normality
Age	35.2	12.5	18	65	0.15	3.2	0.98
Gender	0.52	0.50	0	1	-0.05	3.0	0.99
Marital Status	0.68	0.47	0	1	0.10	3.1	0.98
Education	12.5	2.1	9	16	-0.20	3.3	0.97
Income	1500	500	500	3000	0.30	3.4	0.96
Occupation	1.2	0.8	0	3	-0.10	3.0	0.99
Health Status	0.75	0.42	0	1	0.05	3.1	0.98
Stress Level	2.5	1.0	1	4	0.20	3.2	0.97
Life Satisfaction	3.8	0.9	1	5	-0.15	3.3	0.98
Resilience	2.2	0.8	1	4	0.10	3.1	0.98
Emotional Stability	3.5	0.7	1	5	-0.10	3.2	0.98
Physical Health	4.0	0.6	1	5	-0.05	3.0	0.99
Mental Health	3.2	0.8	1	5	0.15	3.2	0.97
Social Support	2.8	0.9	1	4	0.05	3.1	0.98
Life Events	1.5	0.7	0	3	0.25	3.3	0.96
Personal Growth	3.0	0.8	1	4	-0.10	3.2	0.98
Work-Life Balance	2.5	0.9	1	4	0.15	3.2	0.97
Family Harmony	3.5	0.7	1	5	-0.05	3.0	0.99
Community Involvement	2.0	0.8	0	3	0.10	3.1	0.98
Personal Resilience	2.5	0.9	1	4	0.05	3.1	0.98
Emotional Resilience	3.0	0.8	1	4	-0.10	3.2	0.98
Physical Resilience	3.5	0.7	1	5	-0.05	3.0	0.99
Social Resilience	2.8	0.9	1	4	0.05	3.1	0.98
Life Satisfaction	3.8	0.9	1	5	-0.15	3.3	0.98
Resilience	2.2	0.8	1	4	0.10	3.1	0.98
Emotional Stability	3.5	0.7	1	5	-0.10	3.2	0.98
Physical Health	4.0	0.6	1	5	-0.05	3.0	0.99
Mental Health	3.2	0.8	1	5	0.15	3.2	0.97
Social Support	2.8	0.9	1	4	0.05	3.1	0.98
Life Events	1.5	0.7	0	3	0.25	3.3	0.96
Personal Growth	3.0	0.8	1	4	-0.10	3.2	0.98
Work-Life Balance	2.5	0.9	1	4	0.15	3.2	0.97
Family Harmony	3.5	0.7	1	5	-0.05	3.0	0.99
Community Involvement	2.0	0.8	0	3	0.10	3.1	0.98
Personal Resilience	2.5	0.9	1	4	0.05	3.1	0.98
Emotional Resilience	3.0	0.8	1	4	-0.10	3.2	0.98
Physical Resilience	3.5	0.7	1	5	-0.05	3.0	0.99
Social Resilience	2.8	0.9	1	4	0.05	3.1	0.98

Variable	Mean	Standard deviation	Minimum	Maximum
Age	34.5	10.2	21	55
Gender	0.5	0.5	0	1
Marital status	0.6	0.5	0	1
Education	12.5	1.5	9	16
Income	15.2	5.8	5	35
Health status	0.8	0.4	0	1
Employment status	0.7	0.5	0	1
Home ownership	0.9	0.3	0	1
Vehicle ownership	0.6	0.5	0	1
Life satisfaction	4.2	1.8	1	7
Financial satisfaction	3.8	1.5	1	6
Health satisfaction	4.5	1.2	1	6
Relationship satisfaction	4.1	1.6	1	6
Community satisfaction	3.9	1.4	1	6
Environment satisfaction	3.7	1.3	1	6
Security satisfaction	4.0	1.5	1	6
Quality of life	4.3	1.7	1	7
Overall satisfaction	4.1	1.6	1	6

cgg Arg 190	caa Gln	cct Pro	gcc Ala	aac Asn	aga Arg 195	gaa Glu	gca Ala	aga Arg	cat His	tct Ser 200	tac Tyr	cac His	cgc Arg	tct Ser	cac His 205	746
tat Tyr	gat Asp	cct Pro	cca Pro	cca Pro 210	agc Ser	cga Arg	cag Gln	gct Ala	gga Gly 215	ggt Gly	ctg Leu	tcc Ser	cgc Arg	ttt Phe 220	cct Pro	794
ggg Gly	gcc Ala	aga Arg	agt Ser 225	cac His	cgg Arg	gga Gly	gct Ala 230	ctt Leu	atg Met	gat Asp	tcc Ser	cag Gln	caa Gln 235	gca Ala	tca Ser	842
gga Gly	acc Thr	att Ile 240	gtg Val	caa Gln	att Ile	gtc Val	atc Ile 245	aat Asn	aac Asn	aaa Lys	cac His	aag Lys 250	cat His	gga Gly	caa Gln	890
gtg Val	tgt Cys 255	gtt Val	tcc Ser	aat Asn	gga Gly 260	aag Lys	acc Thr	tat Tyr	tct Ser	cat His	ggc Gly 265	gag Glu	tcc Ser	tgg Trp	cac His	938
cca Pro 270	aac Asn	ctc Leu	cgg Arg	gca Ala	ttt Phe 275	ggc Gly	att Ile	gtg Val	gag Glu	tgt Cys 280	gtg Val	cta Leu	tgt Cys	act Thr	tgt Cys 285	986
aat Asn	gtc Val	acc Thr	aag Lys	caa Gln 290	gag Glu	tgt Cys	aag Lys	aaa Lys	atc Ile 295	cac His	tgc Cys	ccc Pro	aat Asn	cga Arg 300	tac Tyr	1034
ccc Pro	tgc Cys	aag Lys	tat Tyr 305	cct Pro	caa Gln	aaa Lys	ata Ile	gac Asp 310	gga Gly	aag Lys	tgc Cys	tgc Cys	aag Lys 315	gtg Val	tgt Cys	1082
cca Pro	ggt Gly 320	aaa Lys	aaa Lys	gca Ala	aaa Lys	gaa Glu	gaa Glu 325	ctt Leu	cca Pro	ggc Gly	caa Gln	agc Ser 330	ttt Phe	gac Asp	aat Asn	1130
aaa Lys	ggc Gly 335	tac Tyr	ttc Phe	tgc Cys	ggg Gly	gaa Glu 340	gaa Glu	acg Thr	atg Met	cct Pro	gtg Val 345	tat Tyr	gag Glu	tct Ser	gta Val	1178
ttc Phe 350	atg Met	gag Glu	gat Asp	ggg Gly 355	gag Glu	aca Thr	acc Thr	aga Arg	aaa Lys	ata Ile 360	gca Ala	ctg Leu	gag Glu	act Thr	gag Glu 365	1226
aga Arg	cca Pro	cct Pro	cag Gln	gta Val 370	gag Glu	gtc Val	cac His	gtt Val	tgg Trp 375	act Thr	att Ile	cga Arg	aag Lys	ggc Gly 380	att Ile	1274
ctc Leu	cag Gln	cac His	ttc Phe 385	cat His	att Ile	gag Glu	aag Lys	atc Ile 390	tcc Ser	aag Lys	agg Arg	atg Met	ttt Phe 395	gag Glu	gag Glu	1322

FIG. 3C

ctt cct cac ttc aag ctg gtg acc aga aca acc ctg agc cag tgg aag	1370
Leu Pro His Phe Lys Leu Val Thr Arg Thr Thr Leu Ser Gln Trp Lys	
400 405 410	
atc ttc acc gaa gga gaa gct cag atc agc cag atg tgt tca agt cgt	1418
Ile Phe Thr Glu Gly Glu Ala Gln Ile Ser Gln Met Cys Ser Ser Arg	
415 420 425	
gta tgc aga aca gag ctt gaa gat tta gtc aag gtt ttg tac ctg gag	1466
Val Cys Arg Thr Glu Leu Glu Asp Leu Val Lys Val Leu Tyr Leu Glu	
430 435 440 445	
aga tct gaa aag ggc cac tgt taggcaagg	1496
Arg Ser Glu Lys Gly His Cys	
450	

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FIG. 4A

atg gga ggc atg aaa tac atc ttt tgc ttg ttg ttc ttt ctt ttg cta	48
Met Gly Gly Met Lys Tyr Ile Phe Ser Leu Leu Phe Phe Leu Leu Leu	
1 5 10 15	
gaa gga ggc aaa aca gag caa gta aaa cat tca gag aca tat tgc atg	96
Glu Gly Gly Lys Thr Glu Gln Val Lys His Ser Glu Thr Tyr Cys Met	
20 25 30	
ttt caa gac aag aag tac aga gtg ggt gag aga tgg cat cct tac ctg	144
Phe Gln Asp Lys Lys Tyr Arg Val Gly Glu Arg Trp His Pro Tyr Leu	
35 40 45	
gaa cct tat ggg ttg gtt tac tgc gtg aac tgc atc tgc tca gag aat	192
Glu Pro Tyr Gly Leu Val Tyr Cys Val Asn Cys Ile Cys Ser Glu Asn	
50 55 60	
ggg aat gtg ctt tgc agc cga gtc aga tgt cca aat gtt cat tgc ctt	240
Gly Asn Val Leu Cys Ser Arg Val Arg Cys Pro Asn Val His Cys Leu	
65 70 75 80	
tct cct gtg cat att cct cat ctg tgc tgc oct cgc tgc cca gaa gac	288
Ser Pro Val His Ile Pro His Leu Cys Cys Pro Arg Cys Pro Glu Asp	
85 90 95	
tcc tta ccc cca gtg aac aat aag gtg acc agc aag tct tgc gag tac	336
Ser Leu Pro Val Asn Asn Lys Val Thr Ser Lys Ser Cys Glu Tyr	
100 105 110	
aat ggg aca act tac caa cat gga gag ctg ttc gta gct gaa ggg ctc	384
Asn Gly Thr Thr Tyr Gln His Gly Glu Leu Phe Val Ala Glu Gly Leu	
115 120 125	
ttt cag aat cgg caa ccc aat caa tgc acc cag tgc agc tgt tgc gag	432
Phe Gln Asn Arg Gln Pro Asn Gln Cys Thr Gln Cys Ser Cys Ser Glu	
130 135 140	
gga aac gtg tat tgt ggt ctc aag act tgc ccc aaa tta acc tgt gcc	480
Gly Asn Val Tyr Cys Gly Leu Lys Thr Cys Pro Lys Leu Thr Cys Ala	
145 150 155 160	
ttc cca gtc tct gtt cca gat tcc tgc tgc cgg gta tgc aga gga gat	528
Phe Pro Val Ser Val Pro Asp Ser Cys Cys Arg Val Cys Arg Gly Asp	
165 170 175	
gga gaa ctg tca tgg gaa cat tct gat ggt gat atc ttc cgg caa cct	576
Gly Glu Leu Ser Trp Glu His Ser Asp Gly Asp Ile Phe Arg Gln Pro	
180 185 190	
gcc aac aga gaa gca aga cat tct tac cac cgc tct cac tat gat cct	624
Ala Asn Arg Glu Ala Arg His Ser Tyr His Arg Ser His Tyr Asp Pro	
195 200 205	

U.S. Pat. No. 5,811,111

FIG. 4B

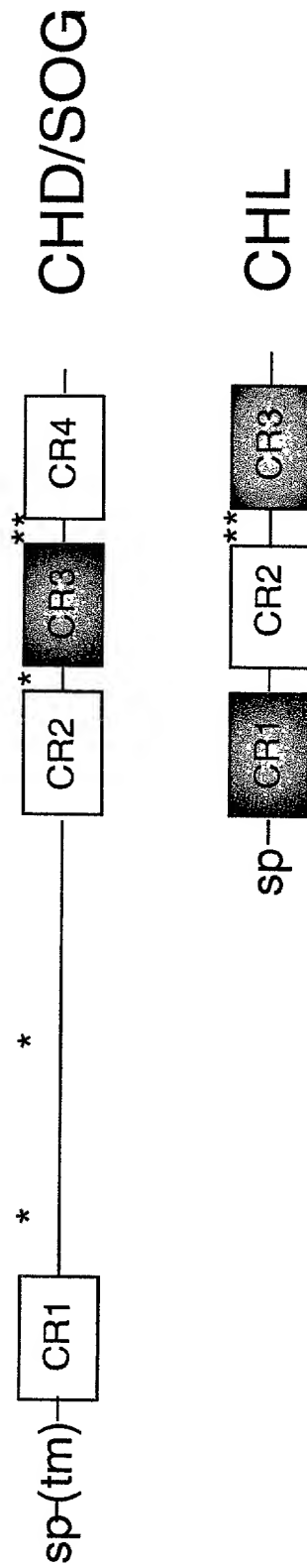
cca	cca	agc	cga	cag	gct	gga	ggt	ctg	tcc	cgc	ttt	cct	ggg	gcc	aga	672
Pro	Pro	Ser	Arg	Gln	Ala	Gly	Gly	Leu	Ser	Arg	Phe	Pro	Gly	Ala	Arg	
210						215					220					
agt	cac	cgg	gga	gct	ctt	atg	gat	tcc	cag	caa	gca	tca	gga	acc	att	720
Ser	His	Arg	Gly	Ala	Leu	Met	Asp	Ser	Gln	Gln	Ala	Ser	Gly	Thr	Ile	
225					230					235					240	
gtg	caa	att	gtc	atc	aat	aac	aaa	cac	aag	cat	gga	caa	gtg	tgt	gtt	768
Val	Gln	Ile	Val	Ile	Asn	Asn	Lys	His	Lys	His	Gly	Gln	Val	Cys	Val	
				245					250					255		
tcc	aat	gga	aag	acc	tat	tct	cat	ggc	gag	tcc	tgg	cac	cca	aac	ctc	816
Ser	Asn	Gly	Lys	Thr	Tyr	Ser	His	Gly	Glu	Ser	Trp	His	Pro	Asn	Leu	
			260					265					270			
cgg	gca	ttt	ggc	att	gtg	gag	tgt	gtg	cta	tgt	act	tgt	aat	gtc	acc	864
Arg	Ala	Phe	Gly	Ile	Val	Glu	Cys	Val	Leu	Cys	Thr	Cys	Asn	Val	Thr	
		275					280					285				
aag	caa	gag	tgt	aag	aaa	atc	cac	tgc	ccc	aat	cga	tac	ccc	tgc	aag	912
Lys	Gln	Glu	Cys	Lys	Lys	Ile	His	Cys	Pro	Asn	Arg	Tyr	Pro	Cys	Lys	
	290					295					300					
tat	cct	caa	aaa	ata	gac	gga	aag	tgc	tgc	aag	gtg	tgt	cca	gaa	gaa	960
Tyr	Pro	Gln	Lys	Ile	Asp	Gly	Lys	Cys	Cys	Lys	Val	Cys	Pro	Glu	Glu	
305					310					315					320	
ctt	cca	ggc	caa	agc	ttt	gac	aat	aaa	ggc	tac	ttc	tgc	ggg	gaa	gaa	1008
Leu	Pro	Gly	Gln	Ser	Phe	Asp	Asn	Lys	Gly	Tyr	Phe	Cys	Gly	Glu	Glu	
				325					330					335		
acg	atg	cct	gtg	tat	gag	tct	gta	ttc	atg	gag	gat	ggg	gag	aca	acc	1056
Thr	Met	Pro	Val	Tyr	Glu	Ser	Val	Phe	Met	Glu	Asp	Gly	Glu	Thr	Thr	
			340					345					350			
aga	aaa	ata	gca	ctg	gag	act	gag	aga	cca	cct	cag	gta	gag	gtc	cac	1104
Arg	Lys	Ile	Ala	Leu	Glu	Thr	Glu	Arg	Pro	Pro	Gln	Val	Glu	Val	His	
		355					360					365				
gtt	tgg	act	att	cga	aag	ggc	att	ctc	cag	cac	ttc	cat	att	gag	aag	1152
Val	Trp	Thr	Ile	Arg	Lys	Gly	Ile	Leu	Gln	His	Phe	His	Ile	Glu	Lys	
	370					375					380					
atc	tcc	aag	agg	atg	ttt	gag	gag	ctt	cct	cac	ttc	aag	ctg	gtg	acc	1200
Ile	Ser	Lys	Arg	Met	Phe	Glu	Glu	Leu	Pro	His	Phe	Lys	Leu	Val	Thr	
385					390					395					400	
aga	aca	acc	ctg	agc	cag	tgg	aag	atc	ttc	acc	gaa	gga	gaa	gct	cag	1248
Arg	Thr	Thr	Leu	Ser	Gln	Trp	Lys	Ile	Phe	Thr	Glu	Gly	Glu	Ala	Gln	
				405					410					415		

GenBank accession number: U00096.1

1 2

[illegible]

FIG. 5



CR: pro-collagen repeat

sp: signal peptide

tm: transmembrane domain (only in *Drosophila sog*)

*: possible BMP1 cleavage site (A/GD sequence)

FIG. 6A

	1				50
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	MPSLPAPPAP	RLLLGLLLLG	SRPASGTGPE	PPALPIRSEK	EPLPVGAAG
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
	51				100
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	CSFGGKVYAL	DETHWPDLGE	PFGVMRCVLC	ACEAPQWARR	GRGPGRVSCK
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
	101				150
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	NIKPQCPTLA	CRQPRQLPGH	CCQTCPQERS	NLDPQPAGLV	FEYPRDPEHR
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	~~~~~	~~~~~QVAAGH	CCQTCPQERS	SSEKQPSGLS	FEYPRDPEHR
	151				200
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	SYSDRGEPGV	GERTRADGHT	DFVALLTGPR	SQAVARARVS	LLRSSLRFSV
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	SYSDRGEPGA	EERARGDGHT	DFVALLTGPR	SQAVARARAS	LLRSSLRFSI
	201				250
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	SYQRLDRPSR	VRFTDPTGNI	LFEHPATPTQ	DGLVCGVWRA	VPRLSVRLLR
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	SYRRLDRPTR	IRFSDPNGSV	LFEHPAAPTQ	DGLVCGVWRA	VPRLSLRLLR
	251				300
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	AEQLRVALVT	STHPSGEVWG	PLIWQALAA	ETFSAILTLE	DPLQRGVGGI
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	AEQLHVALVT	LTHPSGEVWG	PLIRHRALAA	ETFSAILTLE	GPPQQGVGGI

FIG. 6B

	301				350
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	ALLTSLDTE	SLHFLLLFRG	LL...GGLA	QAPLKLQILH	QGQLLRELQA
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	TLLTSLDTE	SLHFLLLFRG	LLEPRSGGLT	QVPLRLQILH	QGQLLRELQA
	351				400
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	NTSAQEPGFA	EVLPSLTDQE	MDWLELGELQ	MVLEKAGGPE	LRISGYITTR
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	NVSAQEPGFA	EVLPNLTVQE	MDWLVLGELQ	MALEWAGRPG	LRISGHIAAR
	401				450
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	QSCDVLQSVL	CGADALIPVQ	TGAAGSASFI	LLGNGSLIQ	VQVVGTSSEV
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	KSCDVLQSVL	CGADALIPVQ	TGAAGSASLT	LLGNGSLIQ
	451				500
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	VAMTLETKPQ	RKNQRTVLCH	MAGLQPGGHM	AVGMCSSLGA	RGAHMLLQNE
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	AVGICPGLGA	RGAHMLLQNE
	501				550
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	LFLNVGTDKF	PDGELRGHVT	ALCYSGHSAR	YDRLPVPLAG	ALVLPPVRSQ
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	LFLNVGTDKF	PDGELRGHVA	ALPYCGHSAR	HDTLPVPLAG	ALVLPPVKSQ
	551				600
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	AAGHAWLSLD	THCHLHYEVL	LAGLGGSEQG	TVTAHLLGPP	GMPGPQRLLK
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	AAGHAWLSLD	THCHLHYEVL	LAGLGGSEQG	TVTAHLLGPP	GTPGPRRLK

FIG. 6C

	601				650
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	GFYGSEAQGV	VKDLEPVLLR	HLAQGTASLL	ITTKSSPRGE	LRGQVHIASQ
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	GFYGSEAQGV	VKDLEPELLR	HLAKGMASLL	ITTKGSPRGE	LRGQVHIANQ
	651				700
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	CEAGGLRLAS	EGVQMPLAPN	GEAATSPMLP	AGPGPEAPVP	AKHGSPPGRPR
Rchordin	~~~GGLRLAS	EGVRMSLAPN	GEAATSPMLP	AGPGPEAPVP	AKHGSSGRPR
Hchordin	CEVGGLRLEA	AGAEGVRALG	APDPASAAPP	VVPGLPALAP	AKPGGPGRPR
	701				750
Hchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchld5	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	DPNTCFFEGQ	QRPHGARWAP	NYDPLCSLCI	CQRRTVICDP	VVCPPPSCPH
Rchordin	DPNTCFFEGQ	QRPHGARWAP	NYDPLCSLCT	CQRRTVICDP	VVCPPPRCSQ
Hchordin	DPNTCFFEGQ	QRPHGARWAP	NYDPLCSLCT	CQRRTVICDP	VVCPPPSCPH
	751				800
Hchl	~~~~~MGGMKY	IFSLLF.FLL	LEGGKTEQVK	HSETYCMFQD	KKYRVGERWH
Hchld5	~~~~~MGGMKY	IFSLLF.FLL	LEGGKTEQVK	HSETYCMFQD	KKYRVGERWH
Mchl	~~~~~MDGMKY	IISLFFIFVF	LEGSKTEQVK	HSDTYCVFQD	KKYRVGEKWH
Rchl	~~~~~MEGIKY	IASLVFFFVF	LEASKTEPVK	HSETYCMFQD	KKYRVGEKWH
Mchordin	PVQALDQCCP	VCPEKQRSRD	LPSLP.NLEP	GEGCYFDGDR	SWRAAGTRWH
Rchordin	PVQALDQWCP	VCSEKQRSRD	LSSLP.NLEP	GEGCYFDGDR	SWRAAGTRWH
Hchordin	PVQAPDQCCP	VCPEKQDVRD	LPGLPRSRDP	GEGCYFDGDR	SWRAAGTRWH
	801				850
Hchl	PYLEPYGLVY	CVNCIC.SEN	GNVLCSSVRC	PNVHCLSPVH	I.PHLCCPRC
Hchld5	PYLEPYGLVY	CVNCIC.SEN	GNVLCSSVRC	PNVHCLSPVH	I.PHLCCPRC
Mchl	PYLEPYGLVY	CVNCIC.SEN	GNVLCSSVRC	PSLHCLSPVH	I.PHLCCPRC
Rchl	PYLEPYGLVY	CVNCIC.SEN	GNVLCSSVRC	PTLHCLSPVH	I.PHLCCPRC
Mchordin	PVVPPFGLIK	CAVCTCKGAT	GEVHCEKVQC	PRLACAQPVR	ANPTDCKKQC
Rchordin	PVVPPFGLIK	CGVCTCKGVN	GEVHSEKVQC	~~~~~	~~~~~
Hchordin	PVVPPFGLIK	CAVCTCKGGT	GEVHCEKVQC	PRLACAQPVR	VNPTDCKKQC
	851				900
Hchl	PEDSLPPVNN	KVTSKSCEYN	GTTYQHGELE	VAEGLFQNRQ	PNQCTQCSCS
Hchld5	PEDSLPPVNN	KVTSKSCEYN	GTTYQHGELE	VAEGLFQNRQ	PNQCTQCSCS
Mchl	P.DSLPPVNN	KVTSKSCEYN	GTTYQHGELE	IAEGLFQNRQ	PNQCSQCSCS
Rchl	P.DSLPPMNN	KVTSKSCEYN	GTTYQHGELE	IAEGLFQNRQ	PNQCSQCSCS
Mchordin	PVGS..GTNA	KLGDPMQADG	PRGCRFAGQW	FPENQSWHPS	VPPFGEMSCI
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	PVGS..GAHP	QLGDPMQADG	PRGCRFAGQW	FPESQSWHPS	VPPFGEMSCI

FIG. 6D

	901				950
Hchl	EGNVYCGLKT	CPKLTCAFPV	SVPDSCCRVC	RGDGELSWEH	SDGDIFRQPA
Hchld5	EGNVYCGLKT	CPKLTCAFPV	SVPDSCCRVC	RGDGELSWEH	SDGDIFRQPA
Mchl	EGNVYCGLKT	CPKLTCAFPV	SVPDSCCRVC	RGDAELSWEH	ADGDIFRQPA
Rchl	EGNVYCGLKT	CPKLTCAFPV	SVPDSCCRVC	RGDGELSWEH	SDADIFRQPA
Mchordin	TCRCGAGVPH	CERDDCSPL	SCGSGKESRC	CSHCTAQR..	SSETRTLPEL
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	TCRCGAGVPH	CERDDCSLPL	SCGSGKESRC	CSRCTAHRP	APETRTPDEL
	951				1000
Hchl	NREARHSYHR	SHYDPPPSRQ	AGGLSRFPGA	RSHRGALMDS	QQASGTIVQI
Hchld5	NREARHSYHR	SHYDPPPSRQ	AGGLSRFPGA	RSHRGALMDS	QQASGTIVQI
Mchl	NREARHSYLR	SPYDPPPNRQ	AGGLPRFPGS	RSHRGAVIDS	QQASGTIVQI
Rchl	NREARHSYLR	SPYDPPPSRQ	AGGLPRFAGS	RSHRGAVIDS	QQASGTIVQI
Mchordin	EKEAEHS~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	EKEAEGS~~~	~~~~~	~~~~~	~~~~~	~~~~~
	1001				1050
Hchl	VINNKHKHGQ	VCVSNKGTYS	HGESWHPNLR	AFGIVECVLC	TCNVTKQECK
Hchld5	VINNKHKHGQ	VCVSNKGTYS	HGESWHPNLR	AFGIVECVLC	TCNVTKQECK
Mchl	VINNKHKHGQ	VCVSNKGTYS	HGESWHPNLR	AFGIVECVLC	TCNVTKQECK
Rchl	VINNKHKHGQ	VCVSNKGTYS	HGESWHSNLR	AFGIVECVLC	TCNVTKQECK
Mchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
	1051				1100
Hchl	KIHCPNRYPC	KYPQKIDGKC	CKVCPGKKAK	EELPGQSFDN	KGYFCGEETM
Hchld5	KIHCPNRYPC	KYPQKIDGKC	CKVCP.....	EELPGQSFDN	KGYFCGEETM
Mchl	KIHCPNRYPC	KYPQKIDGKC	CKVCPGKKAK	GALAGGPAFG	*~~~~~
Rchl	KIHCPNRYPC	KYPQKLDGKC	CKVCP.....	EEPPSQNFDS	KGSFCGEETM
Mchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
	1101				1150
Hchl	PVYESVFMED	GETTRKIALE	TERPPQVEVH	VWTIRKGILQ	HFHIEKISKR
Hchld5	PVYESVFMED	GETTRKIALE	TERPPQVEVH	VWTIRKGILQ	HFHIEKISKR
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	PVYEAVLVED	GETARKVALE	TEKPPQVVGs	RLDYSKGHSP	ALPH*~~~~~
Mchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
	1151				1200
Hchl	MFEELPHFKL	VTRTTLSQWK	IFTEGEAQIS	QMCSSRVCRT	ELEDLVKVLV
Hchld5	MFEELPHFKL	VTRTTLSQWK	IFTEGEAQIS	QMCSSRVCRT	ELEDLVKVLV
Mchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchl	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Mchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Rchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
Hchordin	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~

FIG. 6E

	1201
Hchl	LERSEKGHC*
Hchld5	LERSEKGHC*
Mchl	~~~~~
Rchl	~~~~~
Mchordin	~~~~~
Rchordin	~~~~~
Hchordin	~~~~~

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FIG. 7A

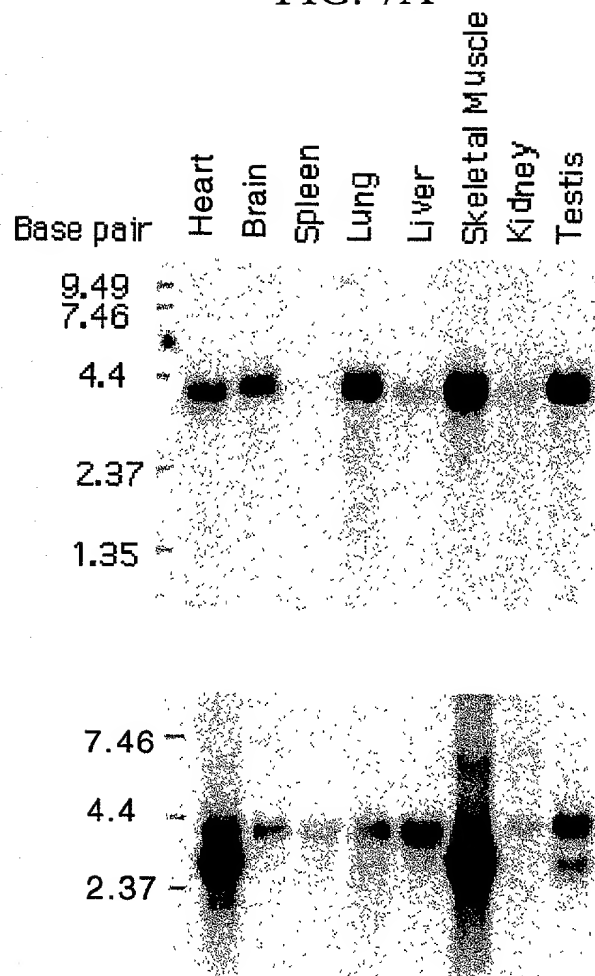
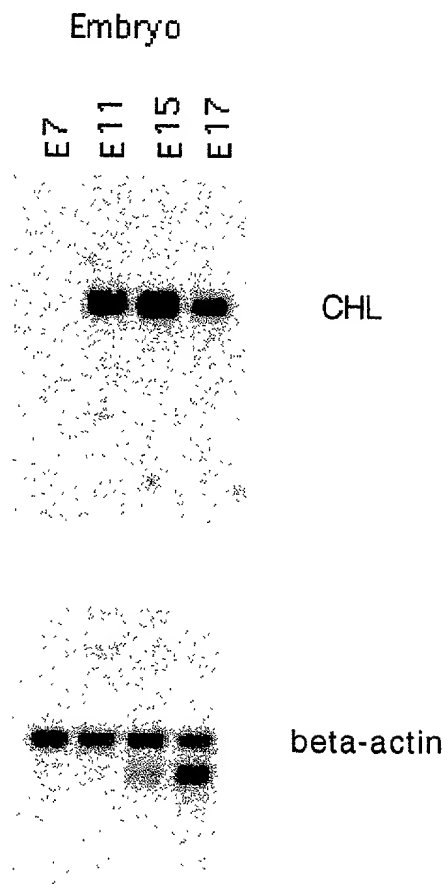


FIG. 7B



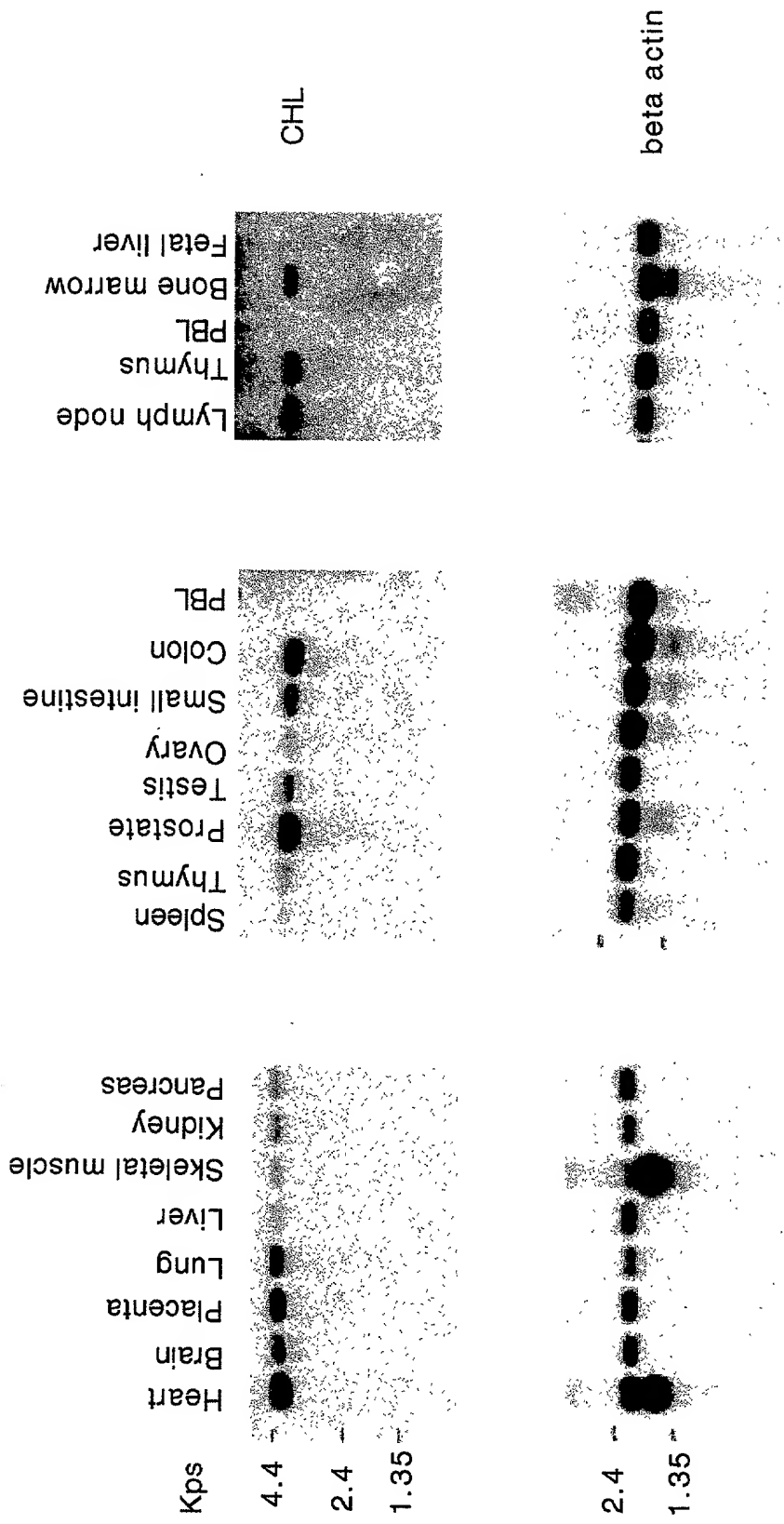
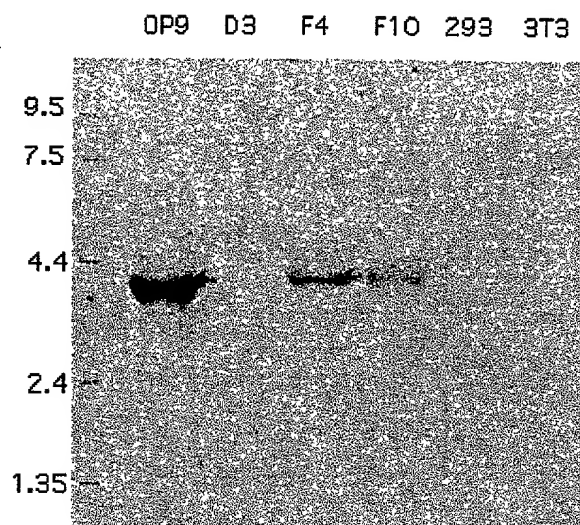


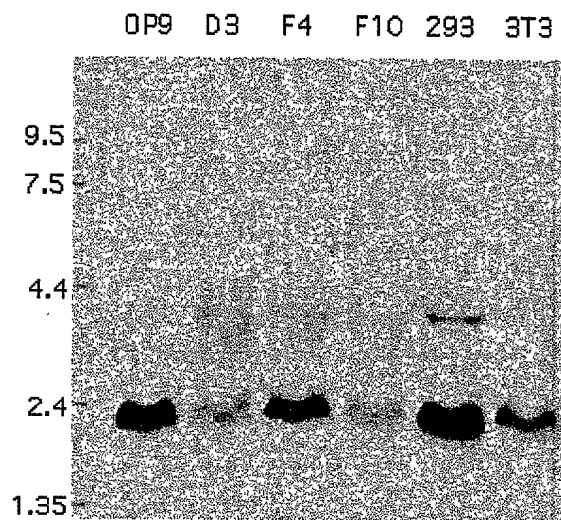
FIG. 8

FIG. 9A



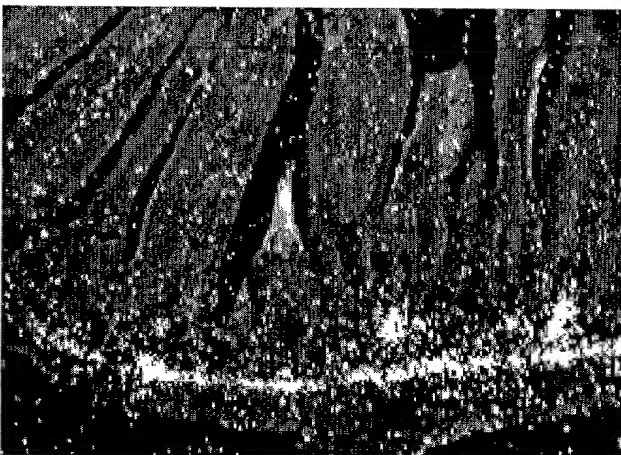
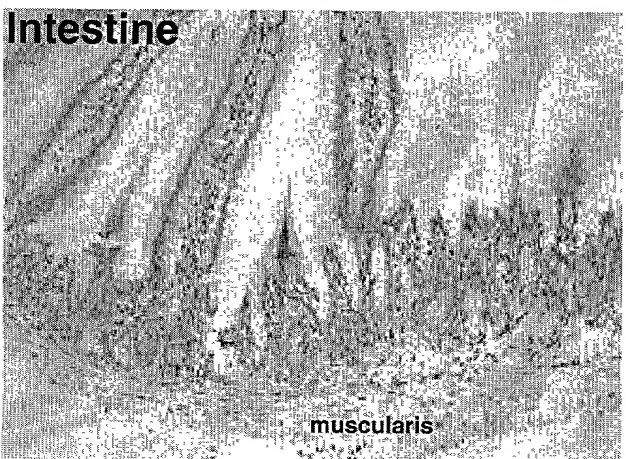
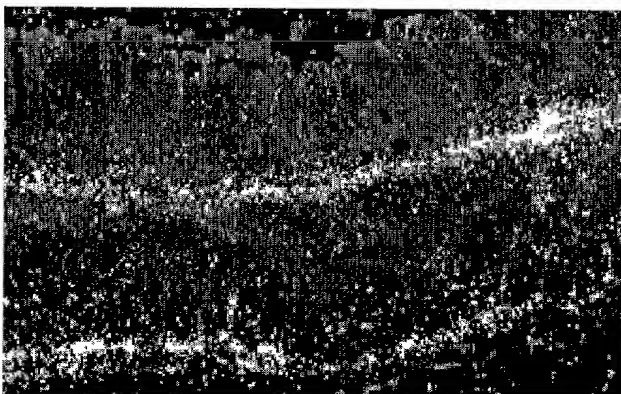
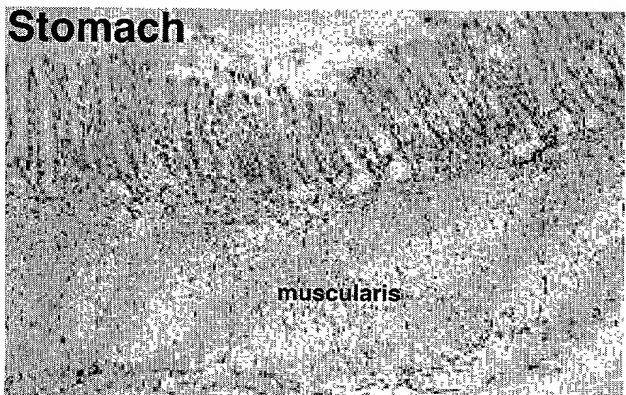
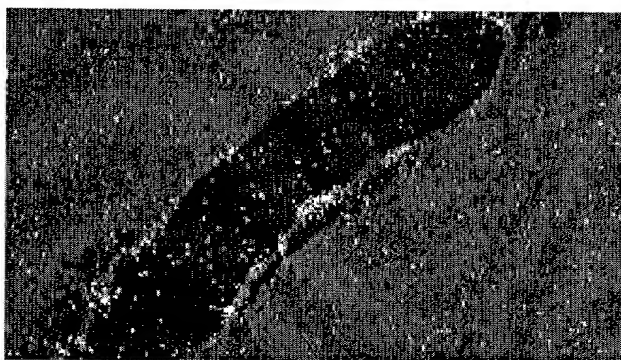
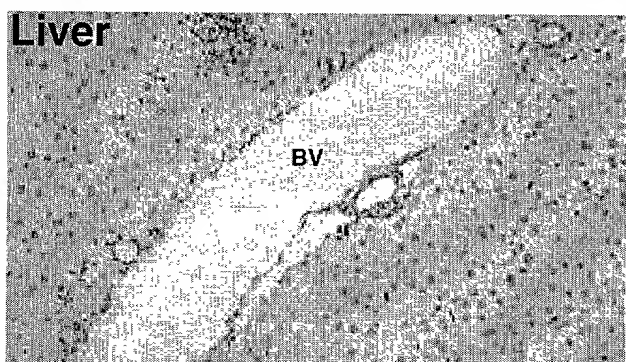
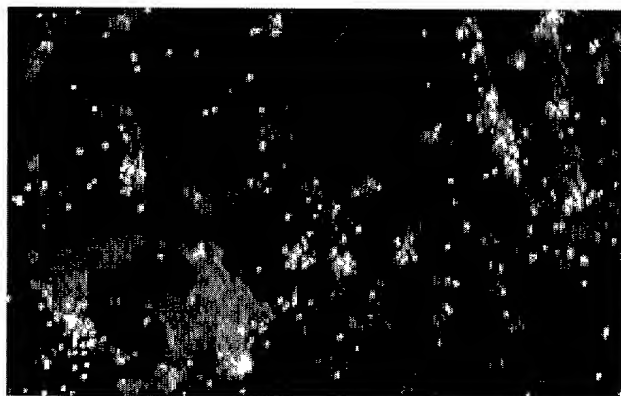
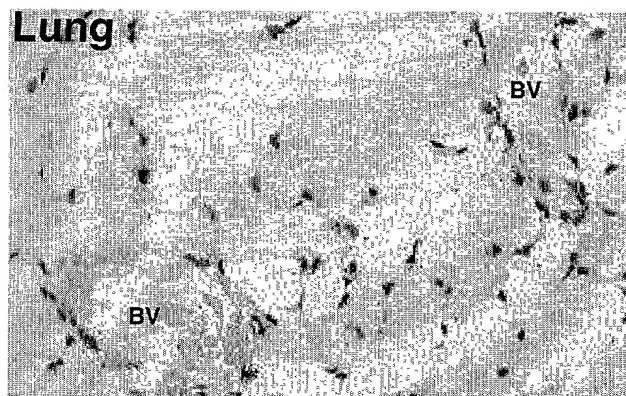
CHL expression

FIG. 9B



beta-actin control

FIG. 10



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FIG. 11

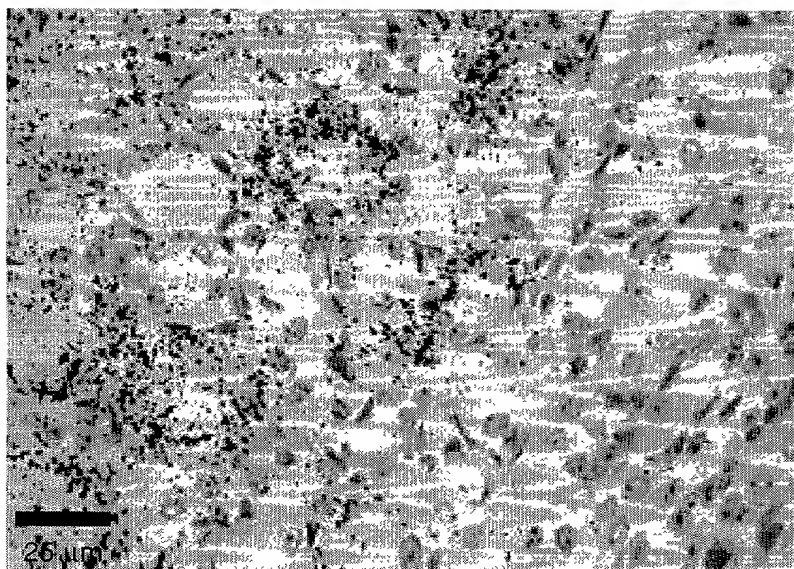
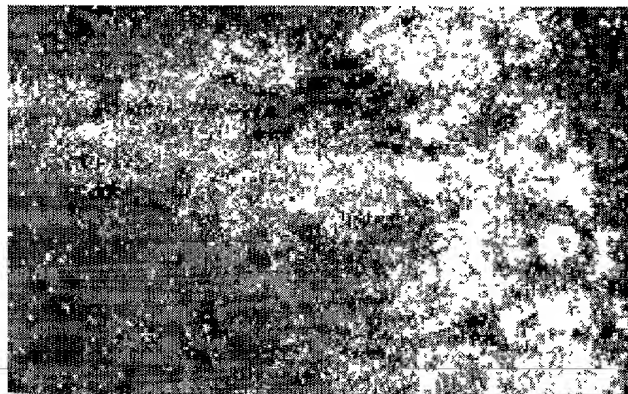
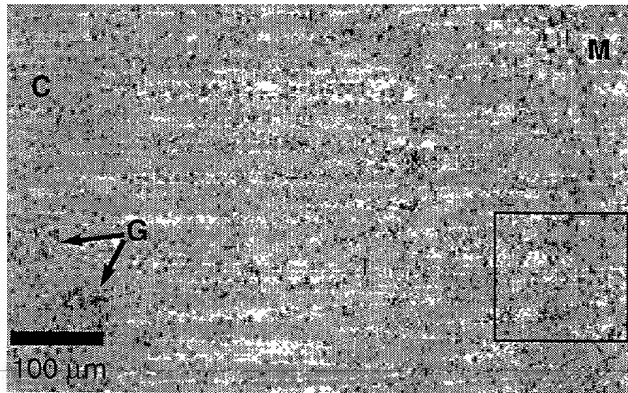
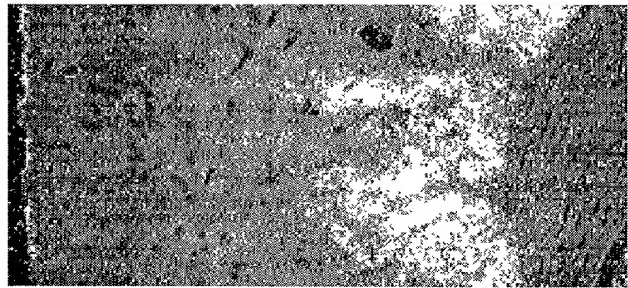
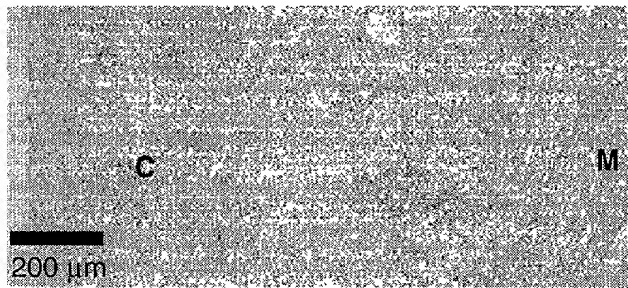
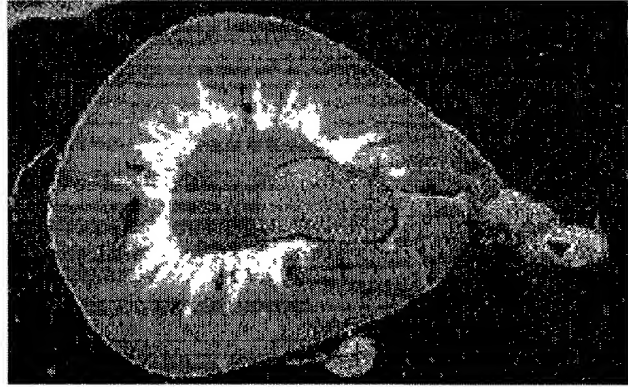
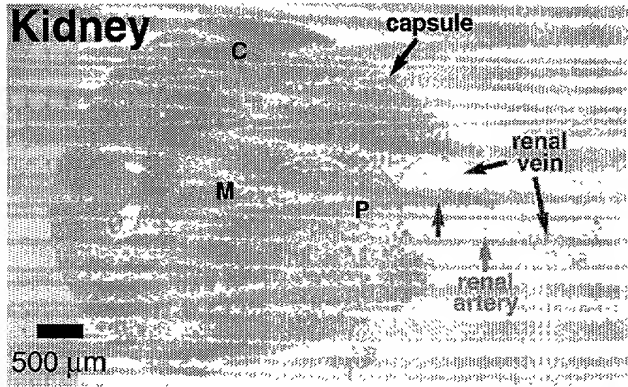


FIG. 12

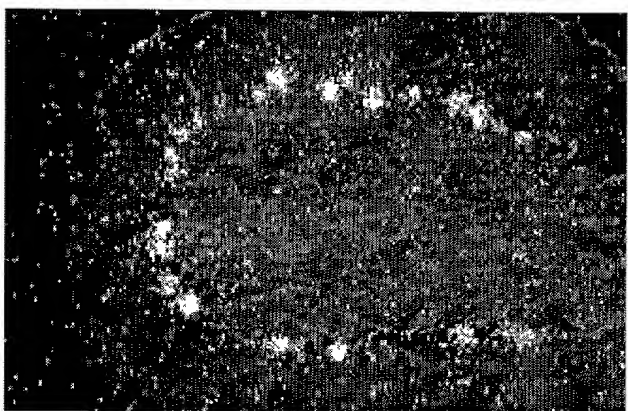
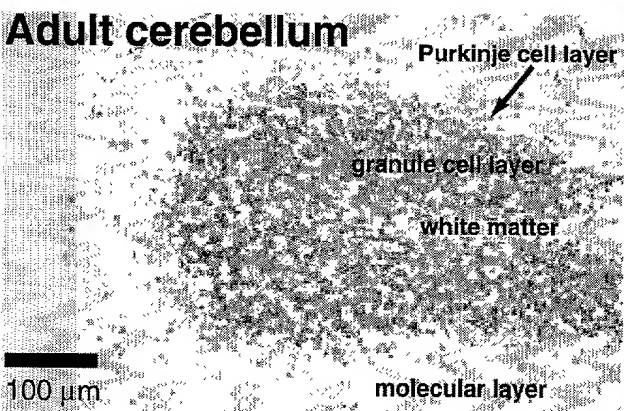
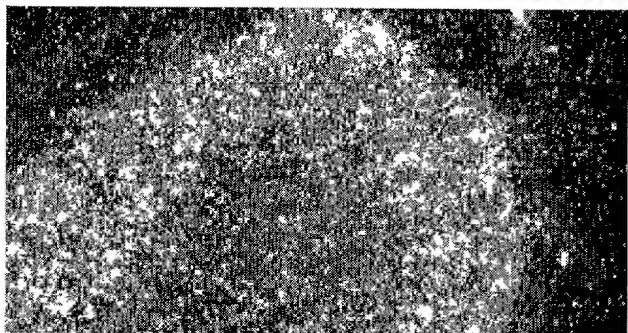
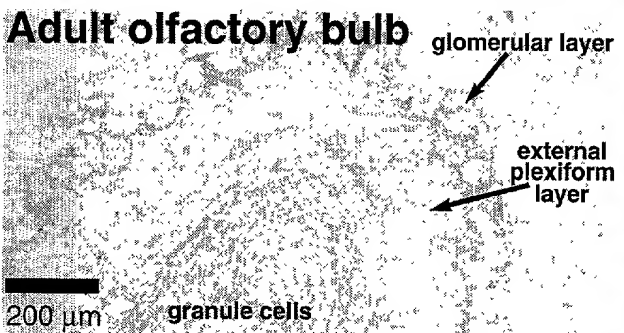
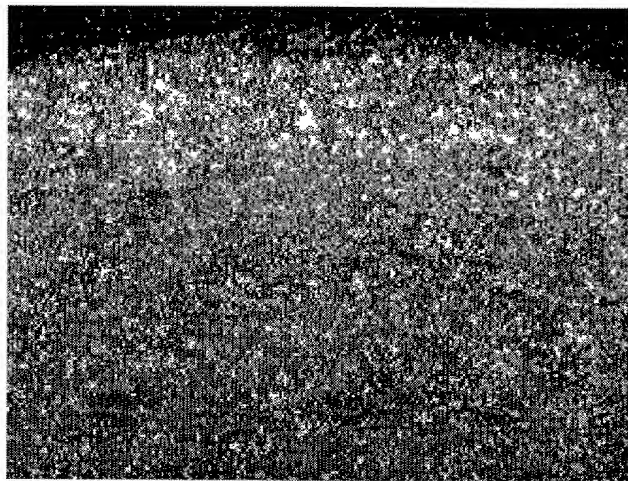
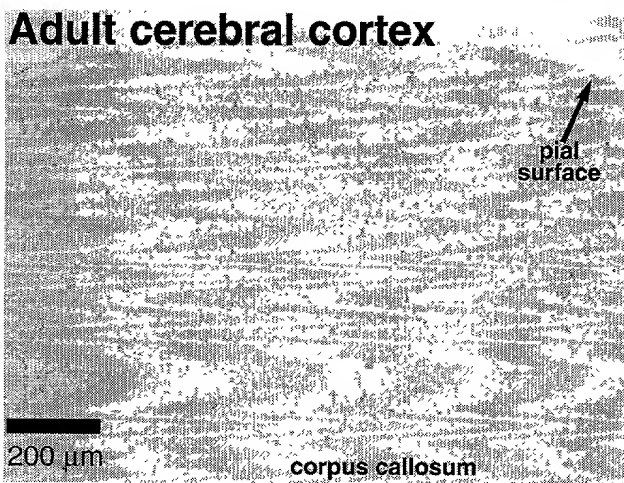
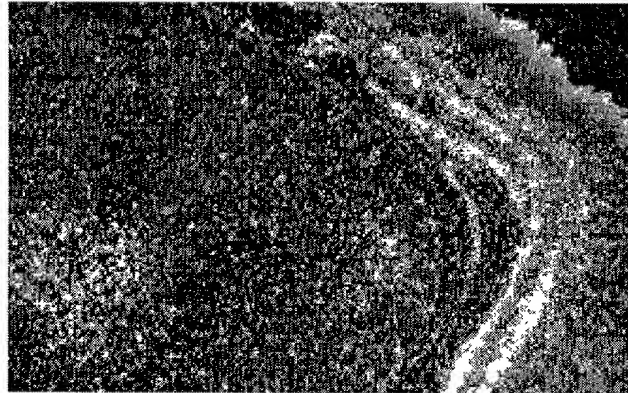
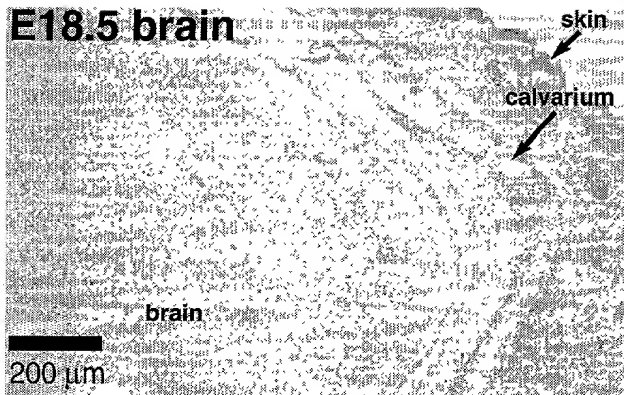


FIG. 13

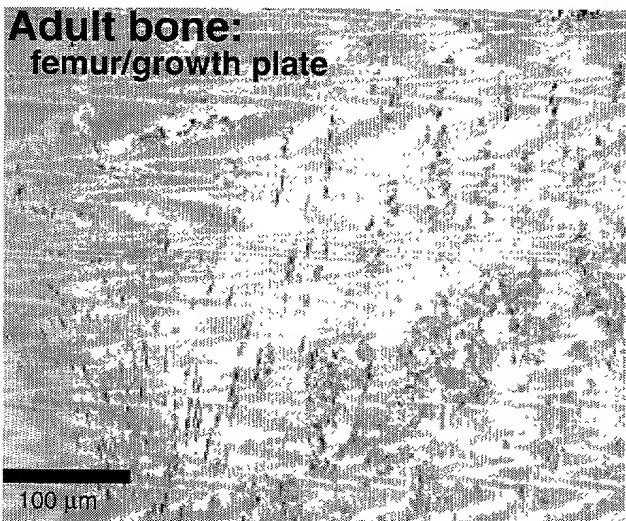
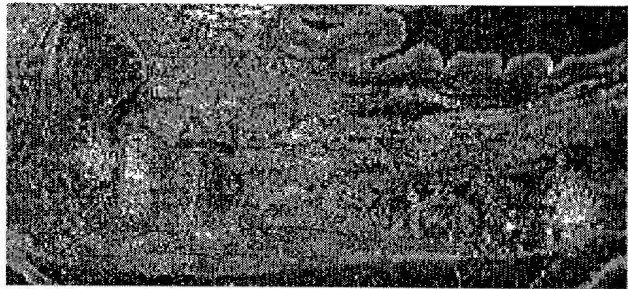
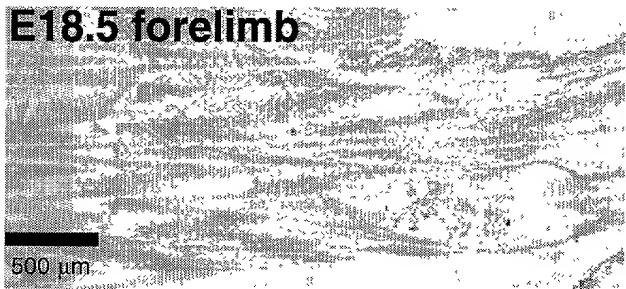
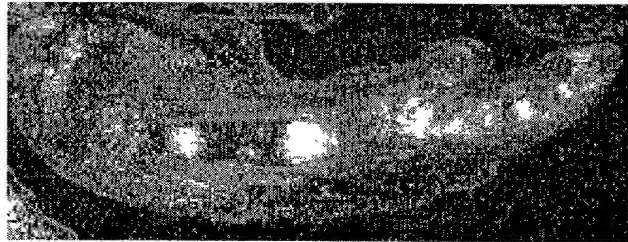
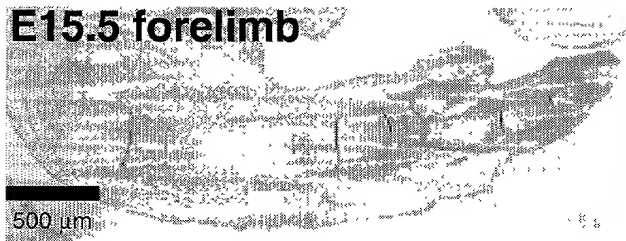
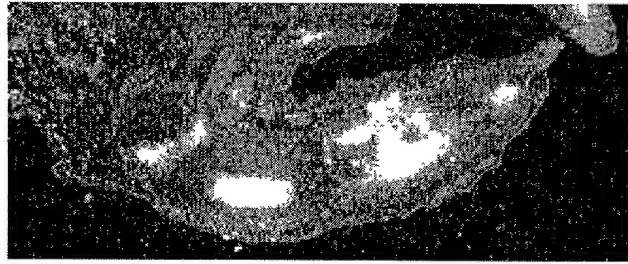
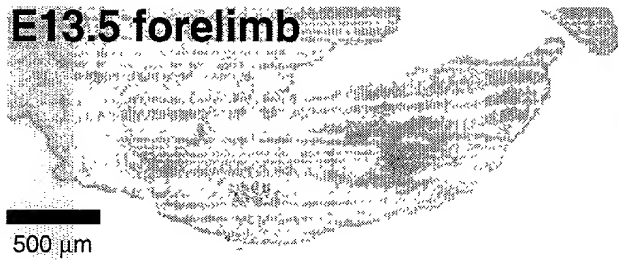


FIG. 14A

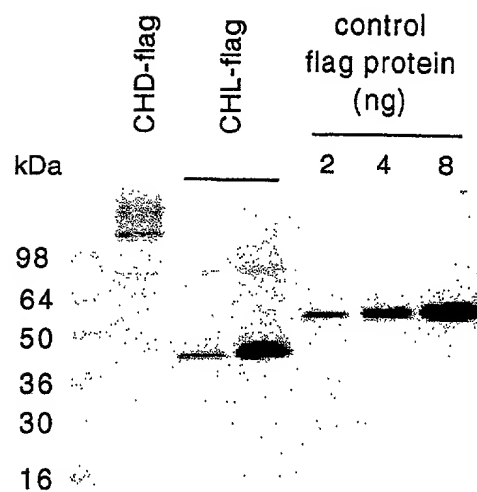


FIG. 14B

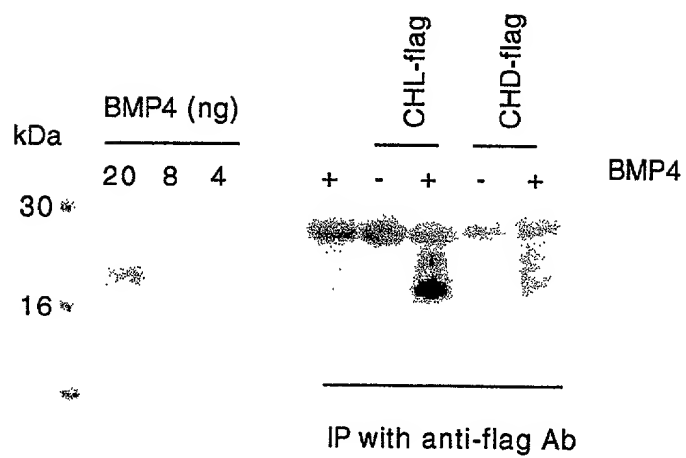


FIG. 14C

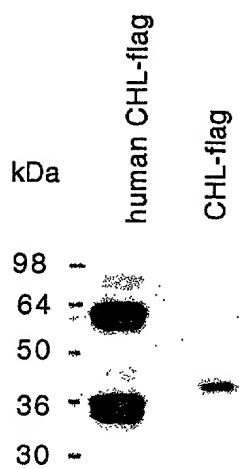


FIG. 15A

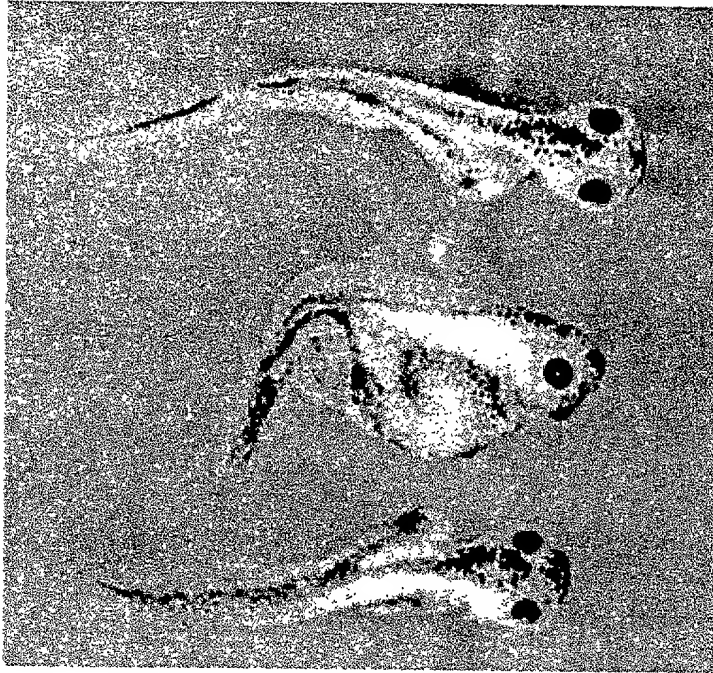


FIG. 15B

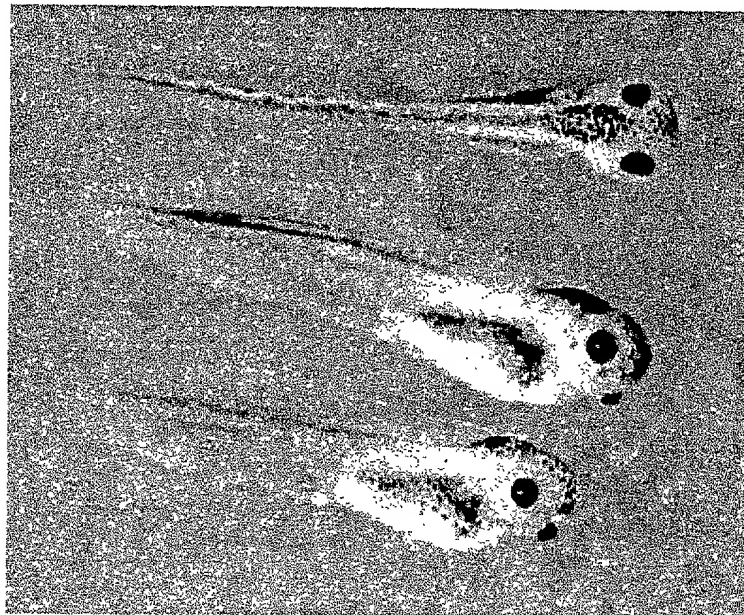


FIG. 16

Inhibitory effect of CHL on the BMP4-dependent erythrocyte generation from ES cells in 7 days

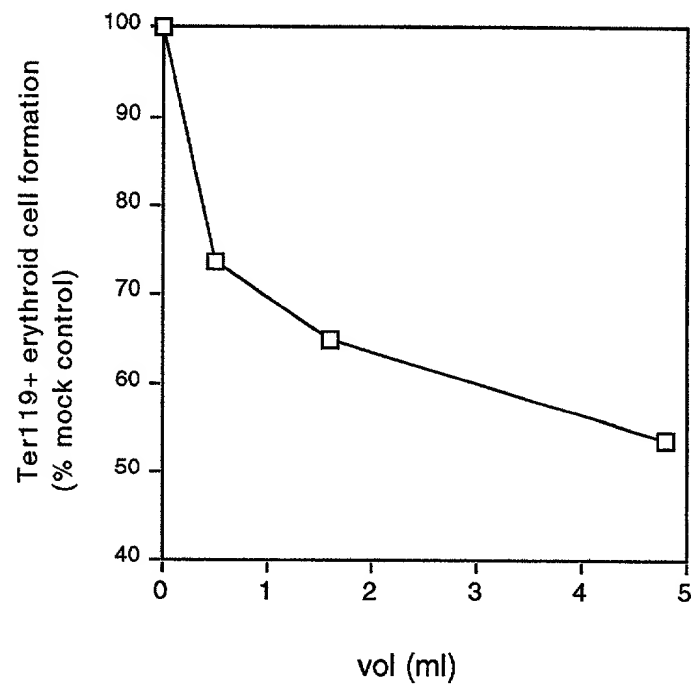


FIG. 17

BMP-4 titration on Bone Marrow Stromal Cell Line A5-F

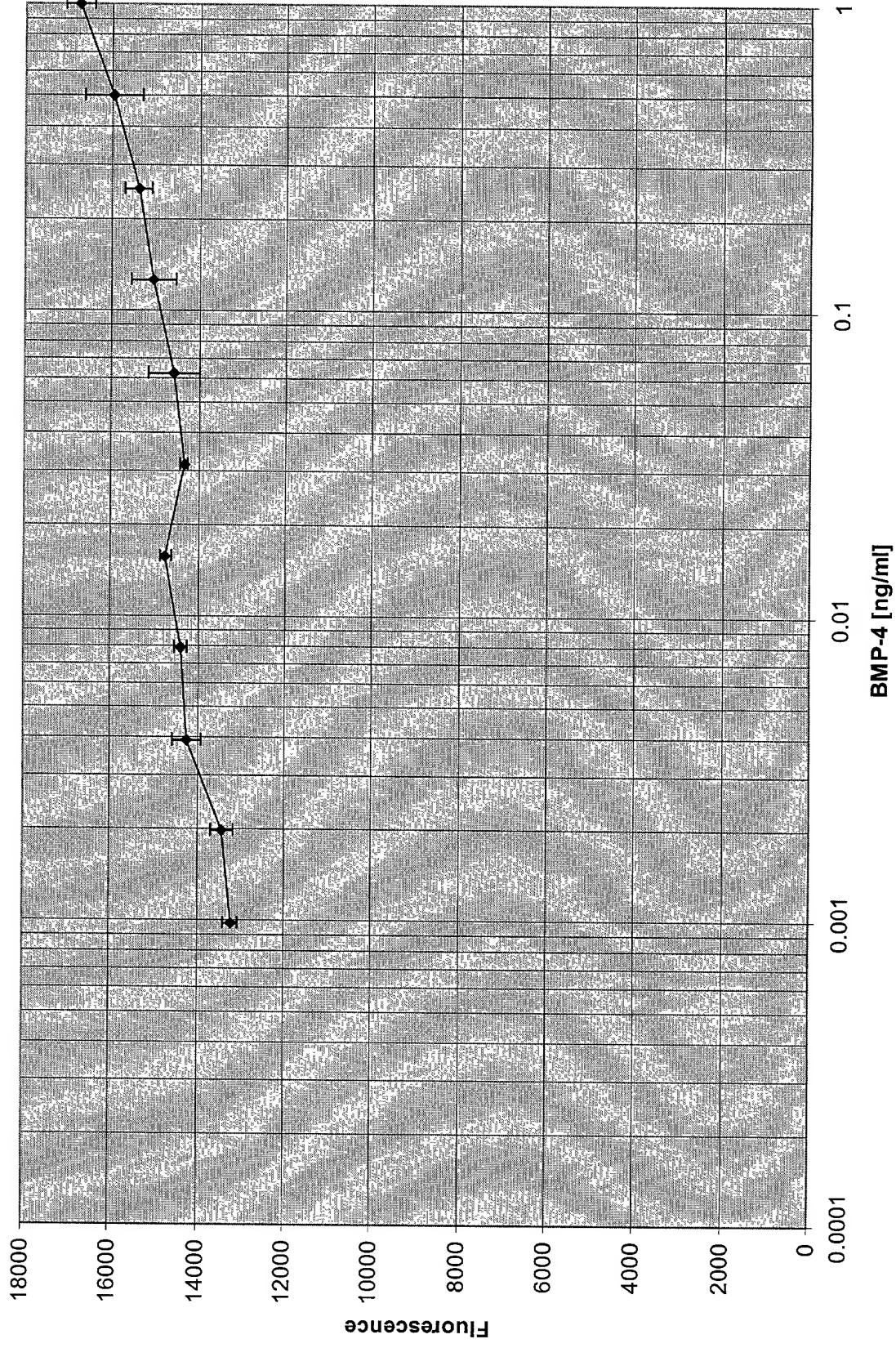
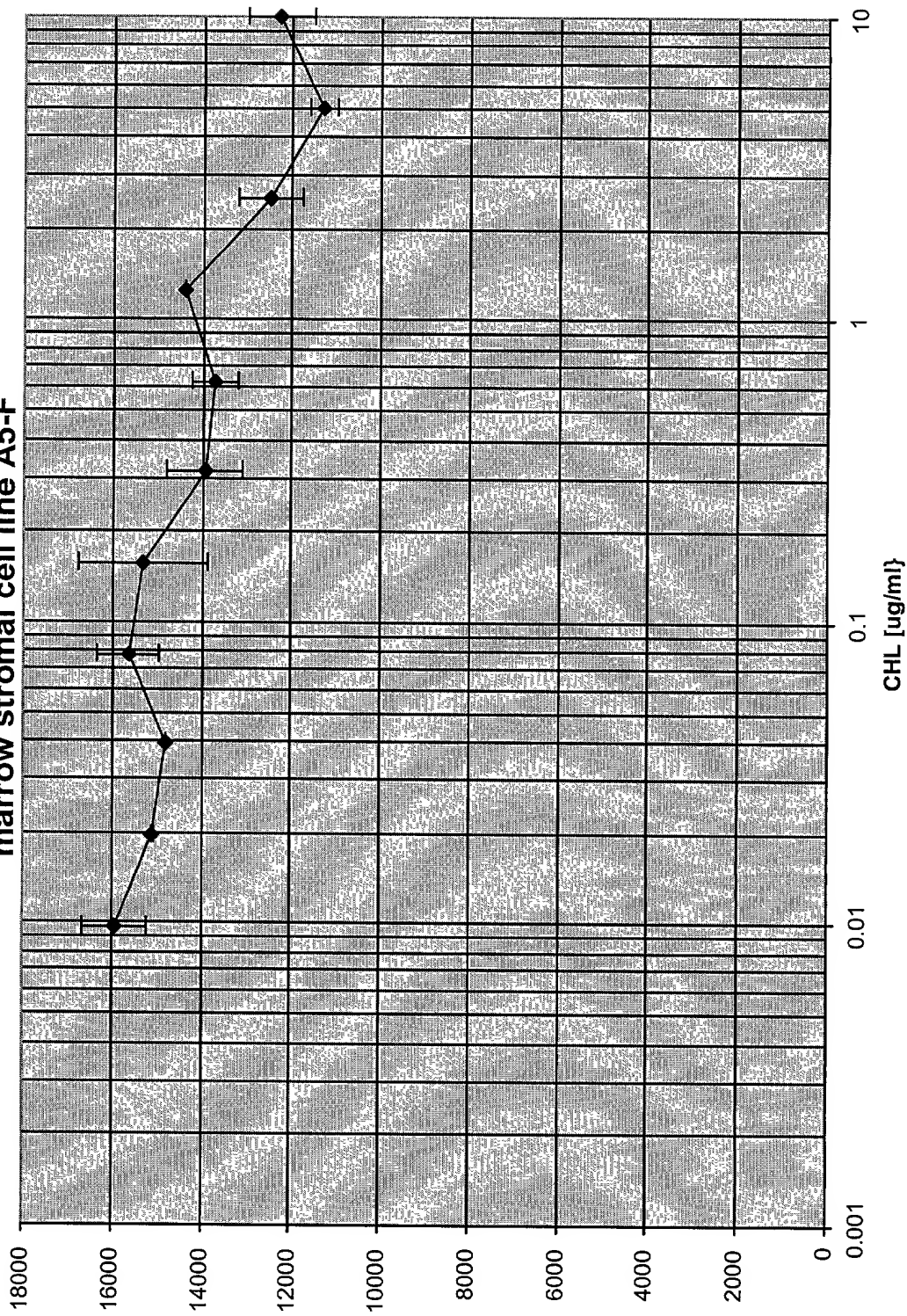


FIG. 18

Chordin-Like Protein titrated in the presence of BMP-4 [0.5ng/ml] on bone marrow stromal cell line A5-F



SEQUENCE LISTING

<110> Nakayama, Naoki
Wen, Duanzhi
Han, Chun-ya
He, Ching
Yu, Dongyin

<120> Chordin-like polypeptides

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gtagctcgaa ggagaaaaaa gtagaag atg gat ggc atg aaa tac atc att tcc 174

Met Asp Gly Met Lys Tyr Ile Ile Ser

1

5

tta ttt ttc atc ttt gtt ttc cta gaa gga agc aaa aca gaa caa gta 222

Leu Phe Phe Ile Phe Val Phe Leu Glu Gly Ser Lys Thr Glu Gln Val

10

15

20

25

aaa cac tca gac aca tat tgc gtg ttt caa gac aag aag tat aga gtg 270

Lys His Ser Asp Thr Tyr Cys Val Phe Gln Asp Lys Lys Tyr Arg Val

30

35

40

ggt gag aaa tgg cat ccc tac ctg gaa ccg tat gga ctg gtt tac tgt 318

Gly Glu Lys Trp His Pro Tyr Leu Glu Pro Tyr Gly Leu Val Tyr Cys

45

50

55

gtg aac tgc atc tgc tct gag aat ggg aat gtg ctt tgc agc cga gtc 366

Val Asn Cys Ile Cys Ser Glu Asn Gly Asn Val Leu Cys Ser Arg Val

60

65

70

aga tgt cca agt ctt cat tgc ctt tca ccc gtg cat att cct cat ctc 414

Arg Cys Pro Ser Leu His Cys Leu Ser Pro Val His Ile Pro His Leu

75

80

85

tgt tgc ccc cgc tgc cca gac tcc tta cca cca gtg aac aat aag gtg	462
Cys Cys Pro Arg Cys Pro Asp Ser Leu Pro Pro Val Asn Asn Lys Val	
90 95 100 105	
acc agc aag tca tgc gaa tac aat gga acc act tac caa cat gga gaa	510
Thr Ser Lys Ser Cys Glu Tyr Asn Gly Thr Thr Tyr Gln His Gly Glu	
110 115 120	
ctg ttc ata gct gaa ggg ctc ttt cag aac cgg caa ccc aat cag tgc	558
Leu Phe Ile Ala Glu Gly Leu Phe Gln Asn Arg Gln Pro Asn Gln Cys	
125 130 135	
agt cag tgt agc tgc tcg gag ggg aat gta tac tgt ggt ctc aag act	606
Ser Gln Cys Ser Cys Ser Glu Gly Asn Val Tyr Cys Gly Leu Lys Thr	
140 145 150	
tgc ccc aaa ctg acc tgt gca ttc cca gtc tct gtt cca gat tct tgc	654
Cys Pro Lys Leu Thr Cys Ala Phe Pro Val Ser Val Pro Asp Ser Cys	
155 160 165	
tgc cga gta tgc aga ggg gat gca gaa tta tcg tgg gaa cat gcg gat	702
Cys Arg Val Cys Arg Gly Asp Ala Glu Leu Ser Trp Glu His Ala Asp	
170 175 180 185	
ggg gat atc ttc cgg caa cct gcc aac aga gaa gca aga cat tct tac	750
Gly Asp Ile Phe Arg Gln Pro Ala Asn Arg Glu Ala Arg His Ser Tyr	
190 195 200	
ctc cgt tcc ccc tac gat cct cca cca aac aga caa gct gga ggt ctt	798
Leu Arg Ser Pro Tyr Asp Pro Pro Pro Asn Arg Gln Ala Gly Gly Leu	
205 210 215	
ccc cgc ttt cct ggg agc aga agt cac cgg gga gct gtt ata gat tcc	846
Pro Arg Phe Pro Gly Ser Arg Ser His Arg Gly Ala Val Ile Asp Ser	
220 225 230	
cag caa gca tcc ggg acc atc gtg cag att gtc atc aat aac aag cac	894
Gln Gln Ala Ser Gly Thr Ile Val Gln Ile Val Ile Asn Asn Lys His	
235 240 245	
aaa cat gga caa gtg tgt gtt tcc aat gga aag acc tac tct cat gga	942
Lys His Gly Gln Val Cys Val Ser Asn Gly Lys Thr Tyr Ser His Gly	
250 255 260 265	
gag tcc tgg cac cca aat cta cga gca ttt ggc att gtg gaa tgt gta	990
Glu Ser Trp His Pro Asn Leu Arg Ala Phe Gly Ile Val Glu Cys Val	
270 275 280	
cta tgc act tgt aat gtc acc aag caa gaa tgt aag aaa atc cac tgc	1038
Leu Cys Thr Cys Asn Val Thr Lys Gln Glu Cys Lys Lys Ile His Cys	
285 290 295	
ccc aat cga tac ccc tgc aag tat cct caa aaa ata gat gga aag tgc	1086
Pro Asn Arg Tyr Pro Cys Lys Tyr Pro Gln Lys Ile Asp Gly Lys Cys	
300 305 310	
tgc aag gtg tgc cca ggt aaa aag gca aaa ggt gca ttg gct gga ggc	1134

Cys Lys Val Cys Pro Gly Lys Lys Ala Lys Gly Ala Leu Ala Gly Gly
 315 320 325

cct gcc ttt ggt tgaatgagat tcacacatag tcctattcag tcttctttgt 1186
 Pro Ala Phe Gly
 330

tcatcaaaac tataaatgac ctgtcttata gttctaacga taatagttct agcaagaatg 1246

aacttcatcc tttcgtcttc tgagacactg atgggttgctt tgaaggaatt aactactcag 1306

agtttctttt gtctacaatg tcaaacacat gccaaagttgc ttatcttggt cttgcttttc 1366

taaattagag agtttacgtt atcactgttt tagaaaaagt cacacctttc atgggtttaa 1426

tcaccaactc acttcaagac ataatccagt actcttttca gatgagatat aaatgagtta 1486

cagtggagag aaattagatt ctgatccaaa tgcacaaat ccacaagtat cttaccccat 1546

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ttaaattttc tgagatatgt tatctgtata tacttatggg gtacagtatg ttaattcaat 1666

acaaatatac aaggtataat tgtcaaatca gggaattat cattctctct cctctgattt 1726

tatccctaga ctcttctagt ctttttaaaa tttatcatca attgggtttt tgatatggta 1786

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Leu Glu Gly Ser Lys Thr Glu Gln Val Lys His Ser Asp Thr Tyr Cys
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Val Phe Gln Asp Lys Lys Tyr Arg Val Gly Glu Lys Trp His Pro Tyr
 35 40 45

Leu Glu Pro Tyr Gly Leu Val Tyr Cys Val Asn Cys Ile Cys Ser Glu
 50 55 60

Asn Gly Asn Val Leu Cys Ser Arg Val Arg Cys Pro Ser Leu His Cys
 65 70 75 80

Leu Ser Pro Val His Ile Pro His Leu Cys Cys Pro Arg Cys Pro Asp
 85 90 95

Ser Leu Pro Pro Val Asn Asn Lys Val Thr Ser Lys Ser Cys Glu Tyr

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Phe Gln Asn Arg Gln Pro Asn Gln Cys Ser Gln Cys Ser Cys Ser Glu		
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Gly Asn Val Tyr Cys Gly Leu Lys Thr Cys Pro Lys Leu Thr Cys Ala		
145	150	155
Phe Pro Val Ser Val Pro Asp Ser Cys Cys Arg Val Cys Arg Gly Asp		
165	170	175
Ala Glu Leu Ser Trp Glu His Ala Asp Gly Asp Ile Phe Arg Gln Pro		
180	185	190
Ala Asn Arg Glu Ala Arg His Ser Tyr Leu Arg Ser Pro Tyr Asp Pro		
195	200	205
Pro Pro Asn Arg Gln Ala Gly Gly Leu Pro Arg Phe Pro Gly Ser Arg		
210	215	220
Ser His Arg Gly Ala Val Ile Asp Ser Gln Gln Ala Ser Gly Thr Ile		
225	230	235
Val Gln Ile Val Ile Asn Asn Lys His Lys His Gly Gln Val Cys Val		
245	250	255
Ser Asn Gly Lys Thr Tyr Ser His Gly Glu Ser Trp His Pro Asn Leu		
260	265	270
Arg Ala Phe Gly Ile Val Glu Cys Val Leu Cys Thr Cys Asn Val Thr		
275	280	285
Lys Gln Glu Cys Lys Lys Ile His Cys Pro Asn Arg Tyr Pro Cys Lys		
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Tyr Pro Gln Lys Ile Asp Gly Lys Cys Cys Lys Val Cys Pro Gly Lys		
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      Met Gly Gly Met Lys Tyr Ile Phe Ser Leu Leu Phe Phe
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ctt ttg cta gaa gga ggc aaa aca gag caa gta aaa cat tca gag aca 218
Leu Leu Leu Glu Gly Gly Lys Thr Glu Gln Val Lys His Ser Glu Thr
      15             20             25

tat tgc atg ttt caa gac aag aag tac aga gtg ggt gag aga tgg cat 266
Tyr Cys Met Phe Gln Asp Lys Lys Tyr Arg Val Gly Glu Arg Trp His
      30             35             40             45

cct tac ctg gaa cct tat ggg ttg gtt tac tgc gtg aac tgc atc tgc 314
Pro Tyr Leu Glu Pro Tyr Gly Leu Val Tyr Cys Val Asn Cys Ile Cys
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tca gag aat ggg aat gtg ctt tgc agc cga gtc aga tgt cca aat gtt 362
Ser Glu Asn Gly Asn Val Leu Cys Ser Arg Val Arg Cys Pro Asn Val
              65             70             75

cat tgc ctt tct cct gtg cat att cct cat ctg tgc tgc cct cgc tgc 410
His Cys Leu Ser Pro Val His Ile Pro His Leu Cys Cys Pro Arg Cys
              80             85             90

cca gaa gac tcc tta ccc cca gtg aac aat aag gtg acc agc aag tct 458
Pro Glu Asp Ser Leu Pro Pro Val Asn Asn Lys Val Thr Ser Lys Ser
              95             100             105

tgc gag tac aat ggg aca act tac caa cat gga gag ctg ttc gta gct 506
Cys Glu Tyr Asn Gly Thr Thr Tyr Gln His Gly Glu Leu Phe Val Ala
      110             115             120             125

gaa ggg ctc ttt cag aat cgg caa ccc aat caa tgc acc cag tgc agc 554
Glu Gly Leu Phe Gln Asn Arg Gln Pro Asn Gln Cys Thr Gln Cys Ser
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tgt tcg gag gga aac gtg tat tgt ggt ctc aag act tgc ccc aaa tta 602
Cys Ser Glu Gly Asn Val Tyr Cys Gly Leu Lys Thr Cys Pro Lys Leu
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acc tgt gcc ttc cca gtc tct gtt cca gat tcc tgc tgc cgg gta tgc 650
Thr Cys Ala Phe Pro Val Ser Val Pro Asp Ser Cys Cys Arg Val Cys
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aga gga gat gga gaa ctg tca tgg gaa cat tct gat ggt gat atc ttc 698
Arg Gly Asp Gly Glu Leu Ser Trp Glu His Ser Asp Gly Asp Ile Phe
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cgg caa cct gcc aac aga gaa gca aga cat tct tac cac cgc tct cac 746
Arg Gln Pro Ala Asn Arg Glu Ala Arg His Ser Tyr His Arg Ser His
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tat gat cct cca cca agc cga cag gct gga ggt ctg tcc cgc ttt cct 794
Tyr Asp Pro Pro Pro Ser Arg Gln Ala Gly Gly Leu Ser Arg Phe Pro
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Gly Ala Arg Ser His Arg Gly Ala Leu Met Asp Ser Gln Gln Ala Ser	
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Gly Thr Ile Val Gln Ile Val Ile Asn Asn Lys His Lys His Gly Gln	
240 245 250	
gtg tgt gtt tcc aat gga aag acc tat tct cat ggc gag tcc tgg cac	938
Val Cys Val Ser Asn Gly Lys Thr Tyr Ser His Gly Glu Ser Trp His	
255 260 265	
cca aac ctc cgg gca ttt ggc att gtg gag tgt gtg cta tgt act tgt	986
Pro Asn Leu Arg Ala Phe Gly Ile Val Glu Cys Val Leu Cys Thr Cys	
270 275 280 285	
aat gtc acc aag caa gag tgt aag aaa atc cac tgc ccc aat cga tac	1034
Asn Val Thr Lys Gln Glu Cys Lys Lys Ile His Cys Pro Asn Arg Tyr	
290 295 300	
ccc tgc aag tat cct caa aaa ata gac gga aag tgc tgc aag gtg tgt	1082
Pro Cys Lys Tyr Pro Gln Lys Ile Asp Gly Lys Cys Cys Lys Val Cys	
305 310 315	
cca ggt aaa aaa gca aaa gaa gaa ctt cca ggc caa agc ttt gac aat	1130
Pro Gly Lys Lys Ala Lys Glu Glu Leu Pro Gly Gln Ser Phe Asp Asn	
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aaa ggc tac ttc tgc ggg gaa gaa acg atg cct gtg tat gag tct gta	1178
Lys Gly Tyr Phe Cys Gly Glu Glu Thr Met Pro Val Tyr Glu Ser Val	
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Phe Met Glu Asp Gly Glu Thr Thr Arg Lys Ile Ala Leu Glu Thr Glu	
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aga cca cct cag gta gag gtc cac gtt tgg act att cga aag ggc att	1274
Arg Pro Pro Gln Val Glu Val His Val Trp Thr Ile Arg Lys Gly Ile	
370 375 380	
ctc cag cac ttc cat att gag aag atc tcc aag agg atg ttt gag gag	1322
Leu Gln His Phe His Ile Glu Lys Ile Ser Lys Arg Met Phe Glu Glu	
385 390 395	
ctt cct cac ttc aag ctg gtg acc aga aca acc ctg agc cag tgg aag	1370
Leu Pro His Phe Lys Leu Val Thr Arg Thr Thr Leu Ser Gln Trp Lys	
400 405 410	
atc ttc acc gaa gga gaa gct cag atc agc cag atg tgt tca agt cgt	1418
Ile Phe Thr Glu Gly Glu Ala Gln Ile Ser Gln Met Cys Ser Ser Arg	
415 420 425	
gta tgc aga aca gag ctt gaa gat tta gtc aag gtt ttg tac ctg gag	1466
Val Cys Arg Thr Glu Leu Glu Asp Leu Val Lys Val Leu Tyr Leu Glu	
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1496

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 Glu Pro Tyr Gly Leu Val Tyr Cys Val Asn Cys Ile Cys Ser Glu Asn
 50 55 60
 Gly Asn Val Leu Cys Ser Arg Val Arg Cys Pro Asn Val His Cys Leu
 65 70 75 80
 Ser Pro Val His Ile Pro His Leu Cys Cys Pro Arg Cys Pro Glu Asp
 85 90 95
 Ser Leu Pro Pro Val Asn Asn Lys Val Thr Ser Lys Ser Cys Glu Tyr
 100 105 110
 Asn Gly Thr Thr Tyr Gln His Gly Glu Leu Phe Val Ala Glu Gly Leu
 115 120 125
 Phe Gln Asn Arg Gln Pro Asn Gln Cys Thr Gln Cys Ser Cys Ser Glu
 130 135 140
 Gly Asn Val Tyr Cys Gly Leu Lys Thr Cys Pro Lys Leu Thr Cys Ala
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 Phe Pro Val Ser Val Pro Asp Ser Cys Cys Arg Val Cys Arg Gly Asp
 165 170 175
 Gly Glu Leu Ser Trp Glu His Ser Asp Gly Asp Ile Phe Arg Gln Pro
 180 185 190
 Ala Asn Arg Glu Ala Arg His Ser Tyr His Arg Ser His Tyr Asp Pro
 195 200 205
 Pro Pro Ser Arg Gln Ala Gly Gly Leu Ser Arg Phe Pro Gly Ala Arg
 210 215 220
 Ser His Arg Gly Ala Leu Met Asp Ser Gln Gln Ala Ser Gly Thr Ile
 225 230 235 240
 Val Gln Ile Val Ile Asn Asn Lys His Lys His Gly Gln Val Cys Val

245								250					255				
Ser	Asn	Gly	Lys	Thr	Tyr	Ser	His	Gly	Glu	Ser	Trp	His	Pro	Asn	Leu		
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Lys	Gln	Glu	Cys	Lys	Lys	Ile	His	Cys	Pro	Asn	Arg	Tyr	Pro	Cys	Lys		
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Tyr	Pro	Gln	Lys	Ile	Asp	Gly	Lys	Cys	Cys	Lys	Val	Cys	Pro	Gly	Lys		
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Gln	Val	Glu	Val	His	Val	Trp	Thr	Ile	Arg	Lys	Gly	Ile	Leu	Gln	His		
			370						375						380		
Phe	His	Ile	Glu	Lys	Ile	Ser	Lys	Arg	Met	Phe	Glu	Glu	Leu	Pro	His		
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Val Phe Phe Phe Val Phe Leu Glu Ala Ser Lys Thr Glu Pro Val Lys											
	15				20				25		
cac tca gag aca tat tgc atg ttt caa gac aag aag tat aga gtt ggt											267
His Ser Glu Thr Tyr Cys Met Phe Gln Asp Lys Lys Tyr Arg Val Gly											
	30				35			40			
gag aaa tgg cat ccc tac ctg gaa cca tat gga ctg gtt tac tgt gtg											315
Glu Lys Trp His Pro Tyr Leu Glu Pro Tyr Gly Leu Val Tyr Cys Val											
	45				50			55			
aac tgc atc tgc tca gag aat ggg aat gtg ctt tgc agc cga gtc aga											363
Asn Cys Ile Cys Ser Glu Asn Gly Asn Val Leu Cys Ser Arg Val Arg											
	60				65			70			
tgt cca act ctt cat tgc ctt tca ccc gtg cat att cct cat ctg tgt											411
Cys Pro Thr Leu His Cys Leu Ser Pro Val His Ile Pro His Leu Cys											
	75				80			85			90
tgc ccc cgt tgc cca gac tcc tta cca ccg atg aac aat aag gtg acc											459
Cys Pro Arg Cys Pro Asp Ser Leu Pro Pro Met Asn Asn Lys Val Thr											
	95				100			105			
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Ser Lys Ser Cys Glu Tyr Asn Gly Thr Thr Tyr Gln His Gly Glu Leu											
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Phe Ile Ala Glu Gly Leu Phe Gln Asn Arg Gln Pro Asn Gln Cys Ser											
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Gln Cys Ser Cys Ser Glu Gly Asn Val Tyr Cys Gly Leu Lys Thr Cys											
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Pro Lys Leu Thr Cys Ala Phe Pro Val Ser Val Pro Asp Ser Cys Cys											
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cga gta tgc aga ggg gat gga gaa tta tca tgg gaa cat tct gat gct											699
Arg Val Cys Arg Gly Asp Gly Glu Leu Ser Trp Glu His Ser Asp Ala											
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Asp Ile Phe Arg Gln Pro Ala Asn Arg Glu Ala Arg His Ser Tyr Leu											
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cgt tcc ccc tac gat cct cca cca agc aga caa gct gga ggt ctt cct											795
Arg Ser Pro Tyr Asp Pro Pro Pro Ser Arg Gln Ala Gly Gly Leu Pro											
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Arg Phe Ala Gly Ser Arg Ser His Arg Gly Ala Val Ile Asp Ser Gln											

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Gln Ala Ser Gly Thr Ile Val Gln Ile Val Ile Asn Asn Lys His Lys			
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cat gga caa gtg tgt gtt tcc aat gga aag acc tat tct cac gga gaa			939
His Gly Gln Val Cys Val Ser Asn Gly Lys Thr Tyr Ser His Gly Glu			
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tcc tgg cat tca aat cta cga gct ttt ggc att gtg gaa tgt gtt cta			987
Ser Trp His Ser Asn Leu Arg Ala Phe Gly Ile Val Glu Cys Val Leu			
	270	275	280
tgc act tgt aat gtc acc aag caa gaa tgt aag aaa atc cac tgc ccc			1035
Cys Thr Cys Asn Val Thr Lys Gln Glu Cys Lys Lys Ile His Cys Pro			
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Lys Val Cys Pro Glu Glu Pro Pro Ser Gln Asn Phe Asp Ser Lys Gly			
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Ser Phe Cys Gly Glu Glu Thr Met Pro Val Tyr Glu Ala Val Leu Val			
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Glu Asp Gly Glu Thr Ala Arg Lys Val Ala Leu Glu Thr Glu Lys Pro			
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cct caa gta gta ggt tca cgt ttg gac tat tcg aaa ggg cat tct cca			1275
Pro Gln Val Val Gly Ser Arg Leu Asp Tyr Ser Lys Gly His Ser Pro			
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Ala Leu Pro His			
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Pro Pro Ser Arg Gln Ala Gly Gly Leu Pro Arg Phe Ala Gly Ser Arg
210 215 220

Ser His Arg Gly Ala Val Ile Asp Ser Gln Gln Ala Ser Gly Thr Ile
225 230 235 240

Val Gln Ile Val Ile Asn Asn Lys His Lys His Gly Gln Val Cys Val
245 250 255

Ser Asn Gly Lys Thr Tyr Ser His Gly Glu Ser Trp His Ser Asn Leu
260 265 270

Arg Ala Phe Gly Ile Val Glu Cys Val Leu Cys Thr Cys Asn Val Thr
275 280 285

Lys Gln Glu Cys Lys Lys Ile His Cys Pro Asn Arg Tyr Pro Cys Lys
290 295 300

Tyr Pro Gln Lys Leu Asp Gly Lys Cys Cys Lys Val Cys Pro Glu Glu
305 310 315 320

Pro Pro Ser Gln Asn Phe Asp Ser Lys Gly Ser Phe Cys Gly Glu Glu
325 330 335

Thr Met Pro Val Tyr Glu Ala Val Leu Val Glu Asp Gly Glu Thr Ala
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35 40 45

Ser Arg Val Arg Cys Pro Ser Leu His Cys Leu Ser Pro Val His Ile
50 55 60

Pro His Leu Cys Cys Pro Arg Cys Pro Asp Ser Leu Pro Pro Val Asn
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 1 5 10 15

Tyr Arg Val Gly Glu Lys Trp His Pro Tyr Leu Glu Pro Tyr Gly Leu
 20 25 30

Val Tyr Cys Val Asn Cys Ile Cys Ser Glu Asn Gly Asn Val Leu Cys
 35 40 45

Ser Arg Val Arg Cys Pro Ser Leu His Cys Leu Ser Pro Val His Ile
 50 55 60

Pro His Leu Cys Cys Pro Arg Cys Pro Asp Ser Leu Pro Pro Val Asn
 65 70 75 80

Asn Lys Val Thr Ser Lys Ser Cys Glu Tyr Asn Gly Thr Thr Tyr Gln
 85 90 95

His Gly Glu Leu Phe Ile Ala Glu Gly Leu Phe Gln Asn Arg Gln Pro
 100 105 110

Asn Gln Cys Ser Gln Cys Ser Cys Ser Glu Gly Asn Val Tyr Cys Gly
 115 120 125

Leu Lys Thr Cys Pro Lys Leu Thr Cys Ala Phe Pro Val Ser Val Pro
 130 135 140

Asp Ser Cys Cys Arg Val Cys Arg Gly Asp Ala Glu Leu Ser Trp Glu
 145 150 155 160

His Ala Asp Gly Asp Ile Phe Arg Gln Pro Ala Asn Arg Glu Ala Arg
 165 170 175

His Ser Tyr Leu Arg Ser Pro Tyr Asp Pro Pro Pro Asn Arg Gln Ala
 180 185 190

Gly Gly Leu Pro Arg Phe Pro Gly Ser Arg Ser His Arg Gly Ala Val
 195 200 205

Ile Asp Ser Gln Gln Ala Ser Gly Thr Ile Val Gln Ile Val Ile Asn
 210 215 220

Asn Lys His Lys His Gly Gln Val Cys Val Ser Asn Gly Lys Thr Tyr
 225 230 235 240

Ser His Gly Glu Ser Trp His Pro Asn Leu Arg Ala Phe Gly Ile Val
 245 250 255

Glu Cys Val Leu Cys Thr Cys Asn Val Thr Lys Gln Glu Cys Lys Lys
 260 265 270

Ile His Cys Pro Asn Arg Tyr Pro Cys Lys Tyr Pro Gln Lys Ile Asp
 275 280 285

Gly Lys Cys Cys Lys Val Cys Pro Gly Lys Lys Ala Lys Gly Ala Leu
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Ala Gly Gly Pro Ala Phe Gly Gly Ser Gly Thr Asp Tyr Lys Asp Asp
 305 310 315 320

Asp Asp Lys

<210> 10

<211> 962

<212> PRT

<213> Mus musculus

<400> 10

Met Pro Ser Leu Pro Ala Pro Pro Ala Pro Arg Leu Leu Leu Gly Leu
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Leu Leu Leu Gly Ser Arg Pro Ala Ser Gly Thr Gly Pro Glu Pro Pro
 20 25 30

Ala Leu Pro Ile Arg Ser Glu Lys Glu Pro Leu Pro Val Arg Gly Ala
 35 40 45

Ala Gly Cys Ser Phe Gly Gly Lys Val Tyr Ala Leu Asp Glu Thr Trp
 50 55 60

His Pro Asp Leu Gly Glu Pro Phe Gly Val Met Arg Cys Val Leu Cys
 65 70 75 80

Ala Cys Glu Ala Pro Gln Trp Ala Arg Arg Gly Arg Gly Pro Gly Arg
 85 90 95

Val Ser Cys Lys Asn Ile Lys Pro Gln Cys Pro Thr Leu Ala Cys Arg
 100 105 110

Gln Pro Arg Gln Leu Pro Gly His Cys Cys Gln Thr Cys Pro Gln Glu
 115 120 125

Arg Ser Asn Leu Asp Pro Gln Pro Ala Gly Leu Val Phe Glu Tyr Pro
 130 135 140

Arg Asp Pro Glu His Arg Ser Tyr Ser Asp Arg Gly Glu Pro Gly Val
 145 150 155 160

Gly Glu Arg Thr Arg Ala Asp Gly His Thr Asp Phe Val Ala Leu Leu
 165 170 175

Thr Gly Pro Arg Ser Gln Ala Val Ala Arg Ala Arg Val Ser Leu Leu

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485										490					495				
Leu	Phe	Leu	Asn	Val	Gly	Thr	Lys	Asp	Phe	Pro	Asp	Gly	Glu	Leu	Arg				
			500					505					510						
Gly	His	Val	Thr	Ala	Leu	Cys	Tyr	Ser	Gly	His	Ser	Ala	Arg	Tyr	Asp				
		515					520					525							
Arg	Leu	Pro	Val	Pro	Leu	Ala	Gly	Ala	Leu	Val	Leu	Pro	Pro	Val	Arg				
	530					535					540								
Ser	Gln	Ala	Ala	Gly	His	Ala	Trp	Leu	Ser	Leu	Asp	Thr	His	Cys	His				
545					550					555					560				
Leu	His	Tyr	Glu	Val	Leu	Leu	Ala	Gly	Leu	Gly	Gly	Ser	Glu	Gln	Gly				
				565					570					575					
Thr	Val	Thr	Ala	His	Leu	Leu	Gly	Pro	Pro	Gly	Met	Pro	Gly	Pro	Gln				
			580					585					590						
Arg	Leu	Leu	Lys	Gly	Phe	Tyr	Gly	Ser	Glu	Ala	Gln	Gly	Val	Val	Lys				
			595				600					605							
Asp	Leu	Glu	Pro	Val	Leu	Leu	Arg	His	Leu	Ala	Gln	Gly	Thr	Ala	Ser				
	610					615					620								
Leu	Leu	Ile	Thr	Thr	Lys	Ser	Ser	Pro	Arg	Gly	Glu	Leu	Arg	Gly	Gln				
625					630					635					640				
Val	His	Ile	Ala	Ser	Gln	Cys	Glu	Ala	Gly	Gly	Leu	Arg	Leu	Ala	Ser				
				645					650					655					
Glu	Gly	Val	Gln	Met	Pro	Leu	Ala	Pro	Asn	Gly	Glu	Ala	Ala	Thr	Ser				
			660					665					670						
Pro	Met	Leu	Pro	Ala	Gly	Pro	Gly	Pro	Glu	Ala	Pro	Val	Pro	Ala	Lys				
		675					680					685							
His	Gly	Ser	Pro	Gly	Arg	Pro	Arg	Asp	Pro	Asn	Thr	Cys	Phe	Phe	Glu				
	690					695				700									
Gly	Gln	Gln	Arg	Pro	His	Gly	Ala	Arg	Trp	Ala	Pro	Asn	Tyr	Asp	Pro				
705					710					715					720				
Leu	Cys	Ser	Leu	Cys	Ile	Cys	Gln	Arg	Arg	Thr	Val	Ile	Cys	Asp	Pro				
				725					730					735					
Val	Val	Cys	Pro	Pro	Pro	Ser	Cys	Pro	His	Pro	Val	Gln	Ala	Leu	Asp				
			740					745				750							
Gln	Cys	Cys	Pro	Val	Cys	Pro	Glu	Lys	Gln	Arg	Ser	Arg	Asp	Leu	Pro				
		755					760					765							
Ser	Leu	Pro	Asn	Leu	Glu	Pro	Gly	Glu	Gly	Cys	Tyr	Phe	Asp	Gly	Asp				
	770					775					780								
Arg	Ser	Trp	Arg	Ala	Ala	Gly	Thr	Arg	Trp	His	Pro	Val	Val	Pro	Pro				

Asn	Asn	Lys	Val	Thr	Ser	Lys	Ser	Cys	Glu	Tyr	Asn	Gly	Thr	Thr	Tyr	
				85					90					95		
Gln	His	Gly	Glu	Leu	Phe	Val	Ala	Glu	Gly	Leu	Phe	Gln	Asn	Arg	Gln	
			100					105					110			
Pro	Asn	Gln	Cys	Thr	Gln	Cys	Ser	Cys	Ser	Glu	Gly	Asn	Val	Tyr	Cys	
		115					120					125				
Gly	Leu	Lys	Thr	Cys	Pro	Lys	Leu	Thr	Cys	Ala	Phe	Pro	Val	Ser	Val	
	130					135					140					
Pro	Asp	Ser	Cys	Cys	Arg	Val	Cys	Arg	Gly	Asp	Gly	Glu	Leu	Ser	Trp	
145					150					155					160	
Glu	His	Ser	Asp	Gly	Asp	Ile	Phe	Arg	Gln	Pro	Ala	Asn	Arg	Glu	Ala	
			165						170					175		
Arg	His	Ser	Tyr	His	Arg	Ser	His	Tyr	Asp	Pro	Pro	Pro	Ser	Arg	Gln	
			180					185					190			
Ala	Gly	Gly	Leu	Ser	Arg	Phe	Pro	Gly	Ala	Arg	Ser	His	Arg	Gly	Ala	
		195					200					205				
Leu	Met	Asp	Ser	Gln	Gln	Ala	Ser	Gly	Thr	Ile	Val	Gln	Ile	Val	Ile	
	210					215					220					
Asn	Asn	Lys	His	Lys	His	Gly	Gln	Val	Cys	Val	Ser	Asn	Gly	Lys	Thr	
225					230					235					240	
Tyr	Ser	His	Gly	Glu	Ser	Trp	His	Pro	Asn	Leu	Arg	Ala	Phe	Gly	Ile	
			245						250					255		
Val	Glu	Cys	Val	Leu	Cys	Thr	Cys	Asn	Val	Thr	Lys	Gln	Glu	Cys	Lys	
			260					265					270			
Lys	Ile	His	Cys	Pro	Asn	Arg	Tyr	Pro	Cys	Lys	Tyr	Pro	Gln	Lys	Ile	
		275					280					285				
Asp	Gly	Lys	Cys	Cys	Lys	Val	Cys	Pro	Gly	Lys	Lys	Ala	Lys	Glu	Glu	
	290					295					300					
Leu	Pro	Gly	Gln	Ser	Phe	Asp	Asn	Lys	Gly	Tyr	Phe	Cys	Gly	Glu	Glu	
305					310					315					320	
Thr	Met	Pro	Val	Tyr	Glu	Ser	Val	Phe	Met	Glu	Asp	Gly	Glu	Thr	Thr	
			325						330					335		
Arg	Lys	Ile	Ala	Leu	Glu	Thr	Glu	Arg	Pro	Pro	Gln	Val	Glu	Val	His	
			340					345					350			
Val	Trp	Thr	Ile	Arg	Lys	Gly	Ile	Leu	Gln	His	Phe	His	Ile	Glu	Lys	
		355					360					365				
Ile	Ser	Lys	Arg	Met	Phe	Glu	Glu	Leu	Pro	His	Phe	Lys	Leu	Val	Thr	
	370					375					380					

Arg Thr Thr Leu Ser Gln Trp Lys Ile Phe Thr Glu Gly Glu Ala Gln
385 390 395 400

Ile Ser Gln Met Cys Ser Ser Arg Val Cys Arg Thr Glu Leu Glu Asp
405 410 415

Leu Val Lys Val Leu Tyr Leu Glu Arg Ser Glu Lys Gly His Cys
420 425 430

<210> 12

<211> 434

<212> PRT

<213> Homo sapiens

<400> 12

Gly Lys Thr Glu Gln Val Lys His Ser Glu Thr Tyr Cys Met Phe Gln
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Asp Lys Lys Tyr Arg Val Gly Glu Arg Trp His Pro Tyr Leu Glu Pro
20 25 30

Tyr Gly Leu Val Tyr Cys Val Asn Cys Ile Cys Ser Glu Asn Gly Asn
35 40 45

Val Leu Cys Ser Arg Val Arg Cys Pro Asn Val His Cys Leu Ser Pro
50 55 60

Val His Ile Pro His Leu Cys Cys Pro Arg Cys Pro Glu Asp Ser Leu
65 70 75 80

Pro Pro Val Asn Asn Lys Val Thr Ser Lys Ser Cys Glu Tyr Asn Gly
85 90 95

Thr Thr Tyr Gln His Gly Glu Leu Phe Val Ala Glu Gly Leu Phe Gln
100 105 110

Asn Arg Gln Pro Asn Gln Cys Thr Gln Cys Ser Cys Ser Glu Gly Asn
115 120 125

Val Tyr Cys Gly Leu Lys Thr Cys Pro Lys Leu Thr Cys Ala Phe Pro
130 135 140

Val Ser Val Pro Asp Ser Cys Cys Arg Val Cys Arg Gly Asp Gly Glu
145 150 155 160

Leu Ser Trp Glu His Ser Asp Gly Asp Ile Phe Arg Gln Pro Ala Asn
165 170 175

Arg Glu Ala Arg His Ser Tyr His Arg Ser His Tyr Asp Pro Pro Pro
180 185 190

Ser Arg Gln Ala Gly Gly Leu Ser Arg Phe Pro Gly Ala Arg Ser His
195 200 205

Arg Gly Ala Leu Met Asp Ser Gln Gln Ala Ser Gly Thr Ile Val Gln
210 215 220

Ile Val Ile Asn Asn Lys His Lys His Gly Gln Val Cys Val Ser Asn
 225 230 235 240

Gly Lys Thr Tyr Ser His Gly Glu Ser Trp His Pro Asn Leu Arg Ala
 245 250 255

Phe Gly Ile Val Glu Cys Val Leu Cys Thr Cys Asn Val Thr Lys Gln
 260 265 270

Glu Cys Lys Lys Ile His Cys Pro Asn Arg Tyr Pro Cys Lys Tyr Pro
 275 280 285

Gln Lys Ile Asp Gly Lys Cys Cys Lys Val Cys Pro Gly Lys Lys Ala
 290 295 300

Lys Glu Glu Leu Pro Gly Gln Ser Phe Asp Asn Lys Gly Tyr Phe Cys
 305 310 315 320

Gly Glu Glu Thr Met Pro Val Tyr Glu Ser Val Phe Met Glu Asp Gly
 325 330 335

Glu Thr Thr Arg Lys Ile Ala Leu Glu Thr Glu Arg Pro Pro Gln Val
 340 345 350

Glu Val His Val Trp Thr Ile Arg Lys Gly Ile Leu Gln His Phe His
 355 360 365

Ile Glu Lys Ile Ser Lys Arg Met Phe Glu Glu Leu Pro His Phe Lys
 370 375 380

Leu Val Thr Arg Thr Thr Leu Ser Gln Trp Lys Ile Phe Thr Glu Gly
 385 390 395 400

Glu Ala Gln Ile Ser Gln Met Cys Ser Ser Arg Val Cys Arg Thr Glu
 405 410 415

Leu Glu Asp Leu Val Lys Val Leu Tyr Leu Glu Arg Ser Glu Lys Gly
 420 425 430

His Cys

<210> 13
 <211> 360
 <212> PRT
 <213> Rattus norvegicus

<400> 13
 Glu Pro Val Lys His Ser Glu Thr Tyr Cys Met Phe Gln Asp Lys Lys
 1 5 10 15

Tyr Arg Val Gly Glu Lys Trp His Pro Tyr Leu Glu Pro Tyr Gly Leu
 20 25 30

Val Tyr Cys Val Asn Cys Ile Cys Ser Glu Asn Gly Asn Val Leu Cys

35					40					45					
Ser	Arg	Val	Arg	Cys	Pro	Thr	Leu	His	Cys	Leu	Ser	Pro	Val	His	Ile
50					55					60					
Pro	His	Leu	Cys	Cys	Pro	Arg	Cys	Pro	Asp	Ser	Leu	Pro	Pro	Met	Asn
65					70					75					
Asn	Lys	Val	Thr	Ser	Lys	Ser	Cys	Glu	Tyr	Asn	Gly	Thr	Thr	Tyr	Gln
85					90					95					
His	Gly	Glu	Leu	Phe	Ile	Ala	Glu	Gly	Leu	Phe	Gln	Asn	Arg	Gln	Pro
100					105					110					
Asn	Gln	Cys	Ser	Gln	Cys	Ser	Cys	Ser	Glu	Gly	Asn	Val	Tyr	Cys	Gly
115					120					125					
Leu	Lys	Thr	Cys	Pro	Lys	Leu	Thr	Cys	Ala	Phe	Pro	Val	Ser	Val	Pro
130					135					140					
Asp	Ser	Cys	Cys	Arg	Val	Cys	Arg	Gly	Asp	Gly	Glu	Leu	Ser	Trp	Glu
145					150					155					
His	Ser	Asp	Ala	Asp	Ile	Phe	Arg	Gln	Pro	Ala	Asn	Arg	Glu	Ala	Arg
165					170					175					
His	Ser	Tyr	Leu	Arg	Ser	Pro	Tyr	Asp	Pro	Pro	Pro	Ser	Arg	Gln	Ala
180					185					190					
Gly	Gly	Leu	Pro	Arg	Phe	Ala	Gly	Ser	Arg	Ser	His	Arg	Gly	Ala	Val
195					200					205					
Ile	Asp	Ser	Gln	Gln	Ala	Ser	Gly	Thr	Ile	Val	Gln	Ile	Val	Ile	Asn
210					215					220					
Asn	Lys	His	Lys	His	Gly	Gln	Val	Cys	Val	Ser	Asn	Gly	Lys	Thr	Tyr
225					230					235					
Ser	His	Gly	Glu	Ser	Trp	His	Ser	Asn	Leu	Arg	Ala	Phe	Gly	Ile	Val
245					250					255					
Glu	Cys	Val	Leu	Cys	Thr	Cys	Asn	Val	Thr	Lys	Gln	Glu	Cys	Lys	Lys
260					265					270					
Ile	His	Cys	Pro	Asn	Arg	Tyr	Pro	Cys	Lys	Tyr	Pro	Gln	Lys	Leu	Asp
275					280					285					
Gly	Lys	Cys	Cys	Lys	Val	Cys	Pro	Glu	Glu	Pro	Pro	Ser	Gln	Asn	Phe
290					295					300					
Asp	Ser	Lys	Gly	Ser	Phe	Cys	Gly	Glu	Glu	Thr	Met	Pro	Val	Tyr	Glu
305					310					315					
Ala	Val	Leu	Val	Glu	Asp	Gly	Glu	Thr	Ala	Arg	Lys	Val	Ala	Leu	Glu
325					330					335					
Thr	Glu	Lys	Pro	Pro	Gln	Val	Val	Gly	Ser	Arg	Leu	Asp	Tyr	Ser	Lys

340

345

350

Gly His Ser Pro Ala Leu Pro His
 355 360

<210> 14

<211> 20

<212> DNA

<213> Mus musculus

<400> 14

agtgccccagc ttttagtccac

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<210> 15

<211> 20

<212> DNA

<213> Mus musculus

<400> 15

gagatgagga atatgcacgg

20

<210> 16

<211> 18

<212> DNA

<213> Homo sapiens

<400> 16

gacatctgac tcggctgc

18

<210> 17

<211> 17

<212> DNA

<213> Homo sapiens

<400> 17

tcacgcagta aaccaac

17

<210> 18

<211> 948

<212> PRT

<213> Mus musculus

<400> 18

Met Pro Ser Leu Pro Ala Pro Pro Ala Pro Arg Leu Leu Leu Gly Leu
 1 5 10 15

Leu Leu Leu Gly Ser Arg Pro Ala Ser Gly Thr Gly Pro Glu Pro Pro
 20 25 30

Ala Leu Pro Ile Arg Ser Glu Lys Glu Pro Leu Pro Val Arg Gly Ala
 35 40 45

Pro Gly Phe Ala Glu Val Leu Pro Ser Leu Thr Asp Gln Glu Met Asp
 355 360 365
 Trp Leu Glu Leu Gly Glu Leu Gln Met Val Leu Glu Lys Ala Gly Gly
 370 375 380
 Pro Glu Leu Arg Ile Ser Gly Tyr Ile Thr Thr Arg Gln Ser Cys Asp
 385 390 395 400
 Val Leu Gln Ser Val Leu Cys Gly Ala Asp Ala Leu Ile Pro Val Gln
 405 410 415
 Thr Gly Ala Ala Gly Ser Ala Ser Phe Ile Leu Leu Gly Asn Gly Ser
 420 425 430
 Leu Ile Tyr Gln Val Gln Val Val Gly Thr Gly Ser Glu Val Val Ala
 435 440 445
 Met Thr Leu Glu Thr Lys Pro Gln Arg Lys Asn Gln Arg Thr Val Leu
 450 455 460
 Cys His Met Ala Gly Leu Gln Pro Gly Gly His Met Ala Val Gly Met
 465 470 475 480
 Cys Ser Gly Leu Gly Ala Arg Gly Ala His Met Leu Leu Gln Asn Glu
 485 490 495
 Leu Phe Leu Asn Val Gly Thr Lys Asp Phe Pro Asp Gly Glu Leu Arg
 500 505 510
 Gly His Val Thr Ala Leu Cys Tyr Ser Gly His Ser Ala Arg Tyr Asp
 515 520 525
 Arg Leu Pro Val Pro Leu Ala Gly Ala Leu Val Leu Pro Pro Val Arg
 530 535 540
 Ser Gln Ala Ala Gly His Ala Trp Leu Ser Leu Asp Thr His Cys His
 545 550 555 560
 Leu His Tyr Glu Val Leu Leu Ala Gly Leu Gly Gly Ser Glu Gln Gly
 565 570 575
 Thr Val Thr Ala His Leu Leu Gly Pro Pro Gly Met Pro Gly Pro Gln
 580 585 590
 Arg Leu Leu Lys Gly Phe Tyr Gly Ser Glu Ala Gln Gly Val Val Lys
 595 600 605
 Asp Leu Glu Pro Val Leu Leu Arg His Leu Ala Gln Gly Thr Ala Ser
 610 615 620
 Leu Leu Ile Thr Thr Lys Ser Ser Pro Arg Gly Glu Leu Arg Gly Gln
 625 630 635 640
 Val His Ile Ala Ser Gln Cys Glu Ala Gly Gly Leu Arg Leu Ala Ser
 645 650 655

<210> 19
 <211> 176
 <212> PRT
 <213> Rattus norvegicus

<400> 19
 Gly Gly Leu Arg Leu Ala Ser Glu Gly Val Arg Met Ser Leu Ala Pro
 1 5 10 15
 Asn Gly Glu Ala Ala Thr Ser Pro Met Leu Pro Ala Gly Pro Gly Pro
 20 25 30
 Glu Ala Pro Val Pro Ala Lys His Gly Ser Ser Gly Arg Pro Arg Asp
 35 40 45
 Pro Asn Thr Cys Phe Phe Glu Gly Gln Gln Arg Pro His Gly Ala Arg
 50 55 60
 Trp Ala Pro Asn Tyr Asp Pro Leu Cys Ser Leu Cys Thr Cys Gln Arg
 65 70 75 80
 Arg Thr Val Ile Cys Asp Pro Val Val Cys Pro Pro Pro Arg Cys Ser
 85 90 95
 Gln Pro Val Gln Ala Leu Asp Gln Trp Cys Pro Val Cys Ser Glu Lys
 100 105 110
 Gln Arg Ser Arg Asp Leu Ser Ser Leu Pro Asn Leu Glu Pro Gly Glu
 115 120 125
 Gly Cys Tyr Phe Asp Gly Asp Arg Ser Trp Arg Ala Ala Gly Thr Arg
 130 135 140
 Trp His Pro Val Val Pro Pro Phe Gly Leu Ile Lys Cys Gly Val Cys
 145 150 155 160
 Thr Cys Lys Gly Val Asn Gly Glu Val His Ser Glu Lys Val Gln Cys
 165 170 175

<210> 20
 <211> 801
 <212> PRT
 <213> Homo sapiens

<400> 20
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 Ser Ser Glu Arg Gln Pro Ser Gly Leu Ser Phe Glu Tyr Pro Arg Asp
 20 25 30
 Pro Glu His Arg Ser Tyr Ser Asp Arg Gly Glu Pro Gly Ala Glu Glu

340	345	350
Thr Lys Asp Phe Pro Asp Gly Glu Leu Arg Gly His Val Ala Ala Leu		
355	360	365
Pro Tyr Cys Gly His Ser Ala Arg His Asp Thr Leu Pro Val Pro Leu		
370	375	380
Ala Gly Ala Leu Val Leu Pro Pro Val Lys Ser Gln Ala Ala Gly His		
385	390	395
Ala Trp Leu Ser Leu Asp Thr His Cys His Leu His Tyr Glu Val Leu		
405	410	415
Leu Ala Gly Leu Gly Gly Ser Glu Gln Gly Thr Val Thr Ala His Leu		
420	425	430
Leu Gly Pro Pro Gly Thr Pro Gly Pro Arg Arg Leu Leu Lys Gly Phe		
435	440	445
Tyr Gly Ser Glu Ala Gln Gly Val Val Lys Asp Leu Glu Pro Glu Leu		
450	455	460
Leu Arg His Leu Ala Lys Gly Met Ala Ser Leu Leu Ile Thr Thr Lys		
465	470	475
Gly Ser Pro Arg Gly Glu Leu Arg Gly Gln Val His Ile Ala Asn Gln		
485	490	495
Cys Glu Val Gly Gly Leu Arg Leu Glu Ala Ala Gly Ala Glu Gly Val		
500	505	510
Arg Ala Leu Gly Ala Pro Asp Pro Ala Ser Ala Ala Pro Pro Val Val		
515	520	525
Pro Gly Leu Pro Ala Leu Ala Pro Ala Lys Pro Gly Gly Pro Gly Arg		
530	535	540
Pro Arg Asp Pro Asn Thr Cys Phe Phe Glu Gly Gln Gln Arg Pro His		
545	550	555
Gly Ala Arg Trp Ala Pro Asn Tyr Asp Pro Leu Cys Ser Leu Cys Thr		
565	570	575
Cys Gln Arg Arg Thr Val Ile Cys Asp Pro Val Val Cys Pro Pro Pro		
580	585	590
Ser Cys Pro His Pro Val Gln Ala Pro Asp Gln Cys Cys Pro Val Cys		
595	600	605
Pro Glu Lys Gln Asp Val Arg Asp Leu Pro Gly Leu Pro Arg Ser Arg		
610	615	620
Asp Pro Gly Glu Gly Cys Tyr Phe Asp Gly Asp Arg Ser Trp Arg Ala		
625	630	635
Ala Gly Thr Arg Trp His Pro Val Val Pro Pro Phe Gly Leu Ile Lys		

gctagcggcc gcgccaccat gccgagcctc ccggccccg 39

<210> 24
<211> 36
<212> DNA
<213> Mus musculus

<400> 24
ggatccgtcg acggagtgc cgccttcttt ctccag 36

<210> 25
<211> 1341
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(1341)

<400> 25
atg gga ggc atg aaa tac atc ttt tcg ttg ttg ttc ttt ctt ttg cta 48
Met Gly Gly Met Lys Tyr Ile Phe Ser Leu Leu Phe Phe Leu Leu Leu
1 5 10 15
gaa gga ggc aaa aca gag caa gta aaa cat tca gag aca tat tgc atg 96
Glu Gly Gly Lys Thr Glu Gln Val Lys His Ser Glu Thr Tyr Cys Met
20 25 30
ttt caa gac aag aag tac aga gtg ggt gag aga tgg cat cct tac ctg 144
Phe Gln Asp Lys Lys Tyr Arg Val Gly Glu Arg Trp His Pro Tyr Leu
35 40 45
gaa cct tat ggg ttg gtt tac tgc gtg aac tgc atc tgc tca gag aat 192
Glu Pro Tyr Gly Leu Val Tyr Cys Val Asn Cys Ile Cys Ser Glu Asn
50 55 60
ggg aat gtg ctt tgc agc cga gtc aga tgt cca aat gtt cat tgc ctt 240
Gly Asn Val Leu Cys Ser Arg Val Arg Cys Pro Asn Val His Cys Leu
65 70 75 80
tct cct gtg cat att cct cat ctg tgc tgc cct cgc tgc cca gaa gac 288
Ser Pro Val His Ile Pro His Leu Cys Cys Pro Arg Cys Pro Glu Asp
85 90 95
tcc tta ccc cca gtg aac aat aag gtg acc agc aag tct tgc gag tac 336
Ser Leu Pro Pro Val Asn Asn Lys Val Thr Ser Lys Ser Cys Glu Tyr
100 105 110
aat ggg aca act tac caa cat gga gag ctg ttc gta gct gaa ggg ctc 384
Asn Gly Thr Thr Tyr Gln His Gly Glu Leu Phe Val Ala Glu Gly Leu
115 120 125
ttt cag aat cgg caa ccc aat caa tgc acc cag tgc agc tgt tcg gag 432
Phe Gln Asn Arg Gln Pro Asn Gln Cys Thr Gln Cys Ser Cys Ser Glu
130 135 140

gtt tgg act att cga aag ggc att ctc cag cac ttc cat att gag aag 1152
Val Trp Thr Ile Arg Lys Gly Ile Leu Gln His Phe His Ile Glu Lys
370 375 380

atc tcc aag agg atg ttt gag gag ctt cct cac ttc aag ctg gtg acc 1200
Ile Ser Lys Arg Met Phe Glu Glu Leu Pro His Phe Lys Leu Val Thr
385 390 395 400

aga aca acc ctg agc cag tgg aag atc ttc acc gaa gga gaa gct cag 1248
Arg Thr Thr Leu Ser Gln Trp Lys Ile Phe Thr Glu Gly Glu Ala Gln
405 410 415

atc agc cag atg tgt tca agt cgt gta tgc aga aca gag ctt gaa gat 1296
Ile Ser Gln Met Cys Ser Ser Arg Val Cys Arg Thr Glu Leu Glu Asp
420 425 430

tta gtc aag gtt ttg tac ctg gag aga tct gaa aag ggc cac tgt 1341
Leu Val Lys Val Leu Tyr Leu Glu Arg Ser Glu Lys Gly His Cys
435 440 445

<210> 26
<211> 447
<212> PRT
<213> Homo sapiens

<400> 26
Met Gly Gly Met Lys Tyr Ile Phe Ser Leu Leu Phe Phe Leu Leu Leu
1 5 10 15

Glu Gly Gly Lys Thr Glu Gln Val Lys His Ser Glu Thr Tyr Cys Met
20 25 30

Phe Gln Asp Lys Lys Tyr Arg Val Gly Glu Arg Trp His Pro Tyr Leu
35 40 45

Glu Pro Tyr Gly Leu Val Tyr Cys Val Asn Cys Ile Cys Ser Glu Asn
50 55 60

Gly Asn Val Leu Cys Ser Arg Val Arg Cys Pro Asn Val His Cys Leu
65 70 75 80

Ser Pro Val His Ile Pro His Leu Cys Cys Pro Arg Cys Pro Glu Asp
85 90 95

Ser Leu Pro Pro Val Asn Asn Lys Val Thr Ser Lys Ser Cys Glu Tyr
100 105 110

Asn Gly Thr Thr Tyr Gln His Gly Glu Leu Phe Val Ala Glu Gly Leu
115 120 125

Phe Gln Asn Arg Gln Pro Asn Gln Cys Thr Gln Cys Ser Cys Ser Glu
130 135 140

Gly Asn Val Tyr Cys Gly Leu Lys Thr Cys Pro Lys Leu Thr Cys Ala
145 150 155 160

<212> PRT

<213> Homo sapiens

<400> 27

Glu Gln Val Lys His Ser Glu Thr Tyr Cys Met Phe Gln Asp Lys Lys
1 5 10 15
Tyr Arg Val Gly Glu Arg Trp His Pro Tyr Leu Glu Pro Tyr Gly Leu
20 25 30
Val Tyr Cys Val Asn Cys Ile Cys Ser Glu Asn Gly Asn Val Leu Cys
35 40 45
Ser Arg Val Arg Cys Pro Asn Val His Cys Leu Ser Pro Val His Ile
50 55 60
Pro His Leu Cys Cys Pro Arg Cys Pro Glu Asp Ser Leu Pro Pro Val
65 70 75 80
Asn Asn Lys Val Thr Ser Lys Ser Cys Glu Tyr Asn Gly Thr Thr Tyr
85 90 95
Gln His Gly Glu Leu Phe Val Ala Glu Gly Leu Phe Gln Asn Arg Gln
100 105 110
Pro Asn Gln Cys Thr Gln Cys Ser Cys Ser Glu Gly Asn Val Tyr Cys
115 120 125
Gly Leu Lys Thr Cys Pro Lys Leu Thr Cys Ala Phe Pro Val Ser Val
130 135 140
Pro Asp Ser Cys Cys Arg Val Cys Arg Gly Asp Gly Glu Leu Ser Trp
145 150 155 160
Glu His Ser Asp Gly Asp Ile Phe Arg Gln Pro Ala Asn Arg Glu Ala
165 170 175
Arg His Ser Tyr His Arg Ser His Tyr Asp Pro Pro Pro Ser Arg Gln
180 185 190
Ala Gly Gly Leu Ser Arg Phe Pro Gly Ala Arg Ser His Arg Gly Ala
195 200 205
Leu Met Asp Ser Gln Gln Ala Ser Gly Thr Ile Val Gln Ile Val Ile
210 215 220
Asn Asn Lys His Lys His Gly Gln Val Cys Val Ser Asn Gly Lys Thr
225 230 235 240
Tyr Ser His Gly Glu Ser Trp His Pro Asn Leu Arg Ala Phe Gly Ile
245 250 255
Val Glu Cys Val Leu Cys Thr Cys Asn Val Thr Lys Gln Glu Cys Lys
260 265 270
Lys Ile His Cys Pro Asn Arg Tyr Pro Cys Lys Tyr Pro Gln Lys Ile
275 280 285

Asp Gly Lys Cys Cys Lys Val Cys Pro Glu Glu Leu Pro Gly Gln Ser
 290 295 300

Phe Asp Asn Lys Gly Tyr Phe Cys Gly Glu Glu Thr Met Pro Val Tyr
 305 310 315 320

Glu Ser Val Phe Met Glu Asp Gly Glu Thr Thr Arg Lys Ile Ala Leu
 325 330 335

Glu Thr Glu Arg Pro Pro Gln Val Glu Val His Val Trp Thr Ile Arg
 340 345 350

Lys Gly Ile Leu Gln His Phe His Ile Glu Lys Ile Ser Lys Arg Met
 355 360 365

Phe Glu Glu Leu Pro His Phe Lys Leu Val Thr Arg Thr Thr Leu Ser
 370 375 380

Gln Trp Lys Ile Phe Thr Glu Gly Glu Ala Gln Ile Ser Gln Met Cys
 385 390 395 400

Ser Ser Arg Val Cys Arg Thr Glu Leu Glu Asp Leu Val Lys Val Leu
 405 410 415

Tyr Leu Glu Arg Ser Glu Lys Gly His Cys
 420 425

<210> 28

<211> 429

<212> PRT

<213> Homo sapiens

<400> 28

Gly Lys Thr Glu Gln Val Lys His Ser Glu Thr Tyr Cys Met Phe Gln
 1 5 10 15

Asp Lys Lys Tyr Arg Val Gly Glu Arg Trp His Pro Tyr Leu Glu Pro
 20 25 30

Tyr Gly Leu Val Tyr Cys Val Asn Cys Ile Cys Ser Glu Asn Gly Asn
 35 40 45

Val Leu Cys Ser Arg Val Arg Cys Pro Asn Val His Cys Leu Ser Pro
 50 55 60

Val His Ile Pro His Leu Cys Cys Pro Arg Cys Pro Glu Asp Ser Leu
 65 70 75 80

Pro Pro Val Asn Asn Lys Val Thr Ser Lys Ser Cys Glu Tyr Asn Gly
 85 90 95

Thr Thr Tyr Gln His Gly Glu Leu Phe Val Ala Glu Gly Leu Phe Gln
 100 105 110

Asn Arg Gln Pro Asn Gln Cys Thr Gln Cys Ser Cys Ser Glu Gly Asn

115	120	125
Val Tyr Cys Gly Leu Lys Thr Cys Pro Lys Leu Thr Cys Ala Phe Pro		
130	135	140
Val Ser Val Pro Asp Ser Cys Cys Arg Val Cys Arg Gly Asp Gly Glu		
145	150	155
Leu Ser Trp Glu His Ser Asp Gly Asp Ile Phe Arg Gln Pro Ala Asn		
	165	170
		175
Arg Glu Ala Arg His Ser Tyr His Arg Ser His Tyr Asp Pro Pro Pro		
	180	185
		190
Ser Arg Gln Ala Gly Gly Leu Ser Arg Phe Pro Gly Ala Arg Ser His		
	195	200
		205
Arg Gly Ala Leu Met Asp Ser Gln Gln Ala Ser Gly Thr Ile Val Gln		
	210	215
		220
Ile Val Ile Asn Asn Lys His Lys His Gly Gln Val Cys Val Ser Asn		
225	230	235
		240
Gly Lys Thr Tyr Ser His Gly Glu Ser Trp His Pro Asn Leu Arg Ala		
	245	250
		255
Phe Gly Ile Val Glu Cys Val Leu Cys Thr Cys Asn Val Thr Lys Gln		
	260	265
		270
Glu Cys Lys Lys Ile His Cys Pro Asn Arg Tyr Pro Cys Lys Tyr Pro		
	275	280
		285
Gln Lys Ile Asp Gly Lys Cys Cys Lys Val Cys Pro Glu Glu Leu Pro		
	290	295
		300
Gly Gln Ser Phe Asp Asn Lys Gly Tyr Phe Cys Gly Glu Glu Thr Met		
305	310	315
		320
Pro Val Tyr Glu Ser Val Phe Met Glu Asp Gly Glu Thr Thr Arg Lys		
	325	330
		335
Ile Ala Leu Glu Thr Glu Arg Pro Pro Gln Val Glu Val His Val Trp		
	340	345
		350
Thr Ile Arg Lys Gly Ile Leu Gln His Phe His Ile Glu Lys Ile Ser		
	355	360
		365
Lys Arg Met Phe Glu Glu Leu Pro His Phe Lys Leu Val Thr Arg Thr		
	370	375
		380
Thr Leu Ser Gln Trp Lys Ile Phe Thr Glu Gly Glu Ala Gln Ile Ser		
385	390	395
		400
Gln Met Cys Ser Ser Arg Val Cys Arg Thr Glu Leu Glu Asp Leu Val		
	405	410
		415
Lys Val Leu Tyr Leu Glu Arg Ser Glu Lys Gly His Cys		

420

425

<210> 29
 <211> 38
 <212> DNA
 <213> Homo sapiens

<400> 29
 cggaattcgc caccatggga ggcataaaat acatcttt

38

<210> 30
 <211> 33
 <212> DNA
 <213> Homo sapiens

<400> 30
 cgcgatcca cagtggccct tttcagatct ctc

33

<210> 31
 <211> 936
 <212> PRT
 <213> Mus musculus

<400> 31
 Thr Gly Pro Glu Pro Pro Ala Leu Pro Ile Arg Ser Glu Lys Glu Pro
 1 5 10 15
 Leu Pro Val Arg Gly Ala Ala Gly Cys Ser Phe Gly Gly Lys Val Tyr
 20 25 30
 Ala Leu Asp Glu Thr Trp His Pro Asp Leu Gly Glu Pro Phe Gly Val
 35 40 45
 Met Arg Cys Val Leu Cys Ala Cys Glu Ala Pro Gln Trp Ala Arg Arg
 50 55 60
 Gly Arg Gly Pro Gly Arg Val Ser Cys Lys Asn Ile Lys Pro Gln Cys
 65 70 75 80
 Pro Thr Leu Ala Cys Arg Gln Pro Arg Gln Leu Pro Gly His Cys Cys
 85 90 95
 Gln Thr Cys Pro Gln Glu Arg Ser Asn Leu Asp Pro Gln Pro Ala Gly
 100 105 110
 Leu Val Phe Glu Tyr Pro Arg Asp Pro Glu His Arg Ser Tyr Ser Asp
 115 120 125
 Arg Gly Glu Pro Gly Val Gly Glu Arg Thr Arg Ala Asp Gly His Thr
 130 135 140
 Asp Phe Val Ala Leu Leu Thr Gly Pro Arg Ser Gln Ala Val Ala Arg
 145 150 155 160

Ala	Arg	Val	Ser	Leu	Leu	Arg	Ser	Ser	Leu	Arg	Phe	Ser	Val	Ser	Tyr	165	170	175	
Gln	Arg	Leu	Asp	Arg	Pro	Ser	Arg	Val	Arg	Phe	Thr	Asp	Pro	Thr	Gly	180	185	190	
Asn	Ile	Leu	Phe	Glu	His	Pro	Ala	Thr	Pro	Thr	Gln	Asp	Gly	Leu	Val	195	200	205	
Cys	Gly	Val	Trp	Arg	Ala	Val	Pro	Arg	Leu	Ser	Val	Arg	Leu	Leu	Arg	210	215	220	
Ala	Glu	Gln	Leu	Arg	Val	Ala	Leu	Val	Thr	Ser	Thr	His	Pro	Ser	Gly	225	230	235	240
Glu	Val	Trp	Gly	Pro	Leu	Ile	Trp	Gln	Gly	Ala	Leu	Ala	Ala	Glu	Thr	245	250	255	
Phe	Ser	Ala	Ile	Leu	Thr	Leu	Glu	Asp	Pro	Leu	Gln	Arg	Gly	Val	Gly	260	265	270	
Gly	Ile	Ala	Leu	Leu	Thr	Leu	Ser	Asp	Thr	Glu	Asp	Ser	Leu	His	Phe	275	280	285	
Leu	Leu	Leu	Phe	Arg	Gly	Leu	Leu	Gly	Gly	Leu	Ala	Gln	Ala	Pro	Leu	290	295	300	
Lys	Leu	Gln	Ile	Leu	His	Gln	Gly	Gln	Leu	Leu	Arg	Glu	Leu	Gln	Ala	305	310	315	320
Asn	Thr	Ser	Ala	Gln	Glu	Pro	Gly	Phe	Ala	Glu	Val	Leu	Pro	Ser	Leu	325	330	335	
Thr	Asp	Gln	Glu	Met	Asp	Trp	Leu	Glu	Leu	Gly	Glu	Leu	Gln	Met	Val	340	345	350	
Leu	Glu	Lys	Ala	Gly	Gly	Pro	Glu	Leu	Arg	Ile	Ser	Gly	Tyr	Ile	Thr	355	360	365	
Thr	Arg	Gln	Ser	Cys	Asp	Val	Leu	Gln	Ser	Val	Leu	Cys	Gly	Ala	Asp	370	375	380	
Ala	Leu	Ile	Pro	Val	Gln	Thr	Gly	Ala	Ala	Gly	Ser	Ala	Ser	Phe	Ile	385	390	395	400
Leu	Leu	Gly	Asn	Gly	Ser	Leu	Ile	Tyr	Gln	Val	Gln	Val	Val	Gly	Thr	405	410	415	
Gly	Ser	Glu	Val	Val	Ala	Met	Thr	Leu	Glu	Thr	Lys	Pro	Gln	Arg	Lys	420	425	430	
Asn	Gln	Arg	Thr	Val	Leu	Cys	His	Met	Ala	Gly	Leu	Gln	Pro	Gly	Gly	435	440	445	
His	Met	Ala	Val	Gly	Met	Cys	Ser	Gly	Leu	Gly	Ala	Arg	Gly	Ala	His	450	455	460	

Met	Leu	Leu	Gln	Asn	Glu	Leu	Phe	Leu	Asn	Val	Gly	Thr	Lys	Asp	Phe	
465					470					475					480	
Pro	Asp	Gly	Glu	Leu	Arg	Gly	His	Val	Thr	Ala	Leu	Cys	Tyr	Ser	Gly	
				485					490					495		
His	Ser	Ala	Arg	Tyr	Asp	Arg	Leu	Pro	Val	Pro	Leu	Ala	Gly	Ala	Leu	
			500					505					510			
Val	Leu	Pro	Pro	Val	Arg	Ser	Gln	Ala	Ala	Gly	His	Ala	Trp	Leu	Ser	
		515					520					525				
Leu	Asp	Thr	His	Cys	His	Leu	His	Tyr	Glu	Val	Leu	Leu	Ala	Gly	Leu	
	530					535					540					
Gly	Gly	Ser	Glu	Gln	Gly	Thr	Val	Thr	Ala	His	Leu	Leu	Gly	Pro	Pro	
545				550						555					560	
Gly	Met	Pro	Gly	Pro	Gln	Arg	Leu	Leu	Lys	Gly	Phe	Tyr	Gly	Ser	Glu	
				565					570					575		
Ala	Gln	Gly	Val	Val	Lys	Asp	Leu	Glu	Pro	Val	Leu	Leu	Arg	His	Leu	
			580					585					590			
Ala	Gln	Gly	Thr	Ala	Ser	Leu	Leu	Ile	Thr	Thr	Lys	Ser	Ser	Pro	Arg	
		595					600					605				
Gly	Glu	Leu	Arg	Gly	Gln	Val	His	Ile	Ala	Ser	Gln	Cys	Glu	Ala	Gly	
	610					615					620					
Gly	Leu	Arg	Leu	Ala	Ser	Glu	Gly	Val	Gln	Met	Pro	Leu	Ala	Pro	Asn	
625					630					635					640	
Gly	Glu	Ala	Ala	Thr	Ser	Pro	Met	Leu	Pro	Ala	Gly	Pro	Gly	Pro	Glu	
				645					650					655		
Ala	Pro	Val	Pro	Ala	Lys	His	Gly	Ser	Pro	Gly	Arg	Pro	Arg	Asp	Pro	
			660					665					670			
Asn	Thr	Cys	Phe	Phe	Glu	Gly	Gln	Gln	Arg	Pro	His	Gly	Ala	Arg	Trp	
		675					680					685				
Ala	Pro	Asn	Tyr	Asp	Pro	Leu	Cys	Ser	Leu	Cys	Ile	Cys	Gln	Arg	Arg	
		690				695					700					
Thr	Val	Ile	Cys	Asp	Pro	Val	Val	Cys	Pro	Pro	Pro	Ser	Cys	Pro	His	
705					710					715					720	
Pro	Val	Gln	Ala	Leu	Asp	Gln	Cys	Cys	Pro	Val	Cys	Pro	Glu	Lys	Gln	
				725					730					735		
Arg	Ser	Arg	Asp	Leu	Pro	Ser	Leu	Pro	Asn	Leu	Glu	Pro	Gly	Glu	Gly	
			740					745					750			
Cys	Tyr	Phe	Asp	Gly	Asp	Arg	Ser	Trp	Arg	Ala	Ala	Gly	Thr	Arg	Trp	
		755					760					765				

His Pro Val Val Pro Pro Phe Gly Leu Ile Lys Cys Ala Val Cys Thr
 770 775 780
 Cys Lys Gly Ala Thr Gly Glu Val His Cys Glu Lys Val Gln Cys Pro
 785 790 795 800
 Arg Leu Ala Cys Ala Gln Pro Val Arg Ala Asn Pro Thr Asp Cys Cys
 805 810 815
 Lys Gln Cys Pro Val Gly Ser Gly Thr Asn Ala Lys Leu Gly Asp Pro
 820 825 830
 Met Gln Ala Asp Gly Pro Arg Gly Cys Arg Phe Ala Gly Gln Trp Phe
 835 840 845
 Pro Glu Asn Gln Ser Trp His Pro Ser Val Pro Pro Phe Gly Glu Met
 850 855 860
 Ser Cys Ile Thr Cys Arg Cys Gly Ala Gly Val Pro His Cys Glu Arg
 865 870 875 880
 Asp Asp Cys Ser Pro Pro Leu Ser Cys Gly Ser Gly Lys Glu Ser Arg
 885 890 895
 Cys Cys Ser His Cys Thr Ala Gln Arg Ser Ser Glu Thr Arg Thr Leu
 900 905 910
 Pro Glu Leu Glu Lys Glu Ala Glu His Ser Val Asp Gly Ser Gly Thr
 915 920 925
 Asp Tyr Lys Asp Asp Asp Asp Lys
 930 935